Dreesen L. 2014. Analysis of the intestinal immune response to *Giardia* species in cattle and mice. PhD dissertation.

Analysis of the intestinal immune response to *Giardia* species in cattle and mice

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Dissertation submitted in fulfilment of the requirements for the degree of Doctor (PhD) in Veterinary Sciences

2014

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Voor mijn ouders.

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List of abbreviations

ABCG8	ATP-binding cassette transporter
ACTB	β-actin
ADA	Adenosine deaminase
ANOVA	One-way analysis of variance
AP1	Activator protein 1
ATP	Adenosine triphosphate
CD	Cluster of Differentiation
cDNA	Complementary DNA
CLDN1	Claudin-1
CPG	Cysts per gram
CPT1	Carnitine palmitoyltransferase 1a
DAB	Diaminobenzidin tetrahydrochloride
DBD	DNA-binding domain
DC	Dendritic cell
DNA	Deoxyribonucleic acid
FASN	Fatty acid synthase
GFP	Green fluorescent protein
h	hour
H^+	Hydron, cationic form of atomic hydrogen
H_2O_2	Hydrogen peroxide
HE	Haematoxylin-eosin
HKG	Housekeeping gene
HPF	High power field
HPRT1	Hypoxanthine phosphoribosyltransferase 1
IEL	Intraepithelial lymphocytes
IFA	Immuno-fluorescence assay
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible isoform of nitric oxide synthase
IPA	Ingenuity Pathway Analysis software

KO	Knockout
LBD	Ligand-binding domain
LPS	Lipopolysaccharide
LTi	Lymphoid tissue inducer
M	Molar
mer	Part, denoting polymers
min	Minute
ml	Milliliter
µl	Microliter
µm	Micrometer
mRNA	Messenger ribonucleic acid
n	Number of observations or replicates
Na	Sodium
NF-κB	Nuclear factor-κB
nM	Nanomolar
NO	Nitric oxide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK4	Pyruvate dehydrogenase kinase, isozyme 4
pi	*Post infection*
pIgR	Polymeric immunoglobulin receptor
PLTP	Phospholipid transfer protein
PPAR	Peroxisome proliferator-activated receptor
PPRE	PPAR response elements
qRT-PCR	Quantitative real-time polymerase chain reaction
R	Receptor
RASGRP2	Ras guanyl releasing protein 2
RHOD	Ras homolog gene family member D
RNA	Ribonucleic acid
ROR	RAR-related Orphan Receptor
RPLP0	Ribosomal protein P0
RQI	RNA quality indicator
RXR	9-*cis*-retinoic acid receptor

s	Second (time)
Sd	Standard deviation
SDHA	Succinate dehydrogenase flavoprotein subunit A
SEM	Standard error of the mean
sIg	Secretory immunoglobulin
STAT	Signal Transducers and Activators of Transcription
Ta	Annealing temperature
TAF2	TATA box binding protein (TBP)-associated factor
TGF	Transforming growth factor
Th	helper T cell
TNF	Tumor necrosis factor
tpi	Triosephosphate isomerase
UBE2D2	Ubiquitin-conjugating enzyme E2D2
VICH	International cooperation on harmonization of technical requirements for registration of veterinary products
VSP	Variant-specific surface protein
WHO	World Health Organization
WT	Wild type
xid	x-linked immunodeficient

Chapter 1

Review on *Giardia duodenalis* and infection related host responses

Based on: Giardiosis in mammals: pathogenesis and immunity. Dreesen L. & Geldhof P. Proceedings of the Belgian Royal Academies of Medicine (submitted)

"**U**biquitous" is probably the most appropriate term to introduce the intestinal protozoan parasite *Giardia duodenalis*, which is a primary cause of diarrhea.

First of all, this protozoan does not show a distribution that is limited to a certain area, country or even continent: *Giardia* can be found in hosts all over the globe, both in temperate regions and in parts of the world with a more tropical climate. Secondly, *G. duodenalis* as a species shows no strict host specificity and can infect humans as well as a wide range of animals varying from pets to wild life. Finally, in most of these different host species the prevalence numbers collected for *Giardia* can be fairly high (Feng and Xiao, 2011). This is explained further on in chapter 1, where prevalence numbers are listed.

The latter especially applies to cattle. Finding a *G. duodenalis* free herd is, to say the least, challenging and finding an uninfected calf in a farm where *Giardia* is present is basically impossible. In addition, giardiosis in cattle is characterised by the chronicity of the infection, which translates in faecal cyst excretion for a long duration of time as shown in more detail further on in chapter 1. When a *Giardia* infection in ruminants results in clinical disease, the most common symptom is diarrhea. It is now suspected that *Giardia* has an impact on production in these animals, as was already shown in goat kids and lambs. In cattle, no conclusive data are available on this topic (Geurden et al., 2010), but *Giardia* is considered as a primary cause of ill thrift and diarrhea and thus could potentially lead to significant losses in production.

This introductory chapter will summarize the current knowledge on this intestinal parasite with a particular focus on the pathogenicity, clinical signs and host immune responses observed in different host species.

1.1. Nomenclature and taxonomy of Giardia

Over the years, the nomenclature of the intestinal parasite we now know as *Giardia* has been a subject of controversy and change. The first description of a protozoon resembling *Giardia* dates back to 1681 and was done by the Dutch microscopist Antonie Van Leeuwenhoek whilst examining his own stool (Figure 1.1.) (Dobell, 1920). In 1859, Vilem Lambl "rediscovered" *Giardia* and called it *Cercomonas intestinalis*, unaware that this name was already reserved for another organism. The final genus name *Giardia* was chosen in tribute to a Belgian taxonomist, Alfred Mathieu Giard and first used in 1882 (Boreham et al., 1990). Although this genus name is now well established, the species name *lamblia* is still used interchangeably with *intestinalis* and *duodenalis* in literature. In this thesis, *G. duodenalis* will be used for purposes of consistency.

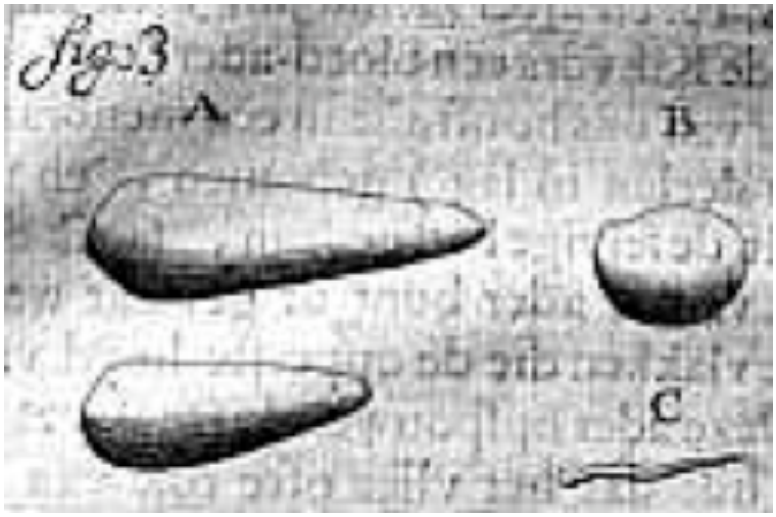

Figure 1.1: The first drawing of *Giardia* by Van Leeuwenhoek

In 1952, after years of host specificity as inspiration for the species name, Filice introduced three major species with a nomenclature based on morphology of the median body, an unique microtubule structure that is important in the formation of the main attachment organelle named the ventral disc. This resulted in the following species: (1) the amphibian species *G. agilis*; (2) the rodent species *G. muris* and (3) the human

species *G. duodenalis*, although organisms of this last group have also been described in other mammals, birds and reptiles. Currently, *G. ardea* (birds), *G. microti* (muskrats and voles) and *G. psittaci* (birds) are accepted as additional species, bringing the total to 6 species (Plutzer et al., 2010).

Further research on *G. duodenalis* using molecular tools revealed that this species is in fact a species complex consisting of eight assemblages (A to H). Some of these assemblages are host-specific while others infect a (limited) range of hosts (Feng and Xiao, 2011). More recently, attempts have been made to re-use species names based on host occurrence (Thompson and Monis, 2004), such as *G. canis* for assemblages C and D found in dogs and *G. bovis* for assemblage E found in lifestock. However, this still remains a suggestion and awaits acceptance by the research community. The established *Giardia* species and *G. duodenalis* subspecies are summarized in **table 1.1**.

Regarding taxonomy, there are two ways to classify *Giardia*. An old system based on morphology would assign *Giardia* to the Phylum Sarcomastigophora, Subphylum mastigophora (=Flagellata), Class zoomastigophorea, Order Diplomonadida and Family Hexamitidae (Morrison et al., 2007). However, based on new genetic, structural and biochemical data *Giardia* belongs to the Phylum Metamonada, Subphylum Trichozoa, Superclass Eopharyngia, Class Trepomonadea, Subclass Diplozoa, Order Giardiia and Family Giardidae (Cavalier-Smith, 2003).

Table 1.1. *Giardia* species and *G. duodenalis* assemblages

Species	Assemblage	Host range	Reference
G. agilis		Amphibians	Kunstler, 1883 (Adam, 2001)
G. ardeae		Birds	Noller, 1920 (Adam, 2001)
G. microti		Muskrats and voles	(Feely, 1988)
G. muris		Rodents	Grassi, 1879 (Adam, 2001)
G. psittaci		Birds	(Erlandsen and Bemrick, 1987)
G. duodenalis	Assemblage A (=*G. duodenalis* sensu stricto?)*	Humans, nonhuman primates, ruminants, alpacas, pigs, horses, canines, cats, ferrets, rodents, marsupials, other mammals	(Karanis and Ey, 1998)
	Assemblage B (=*G. enterica*?)*	Humans, nonhuman primates, cattle, dogs, horses, rabbits, beavers, muskrats	(Homan et al., 1992)
	Assemblage C (= *G. canis*?)*	Domestic and wild canines	(Meloni and Thompson, 1987; Monis et al., 1998)
	Assemblage D (= *G. canis*?)*	Domestic and wild canines	(Monis et al., 1998)
	Assemblage E (= *G. bovis*?)*	Domestic ruminants, pigs	(Ey et al., 1997)
	Assemblage F (=*G. cati*?)*	Cats	(Monis et al., 1999)
	Assemblage G (=*G. simondi*?)*	Mice, rats	(Monis et al., 1999)
	Assemblage H	Marine mammals	(Lasek-Nesselquist et al., 2010)

* Proposed species names, not yet accepted

1.2. Life cycle of Giardia

To complete its simple direct life cycle (Figure 1.2.), *Giardia* appears in two forms: the flagellated trophozoite and the infective cyst stage, both efficiently adapted to survival in different environments (Svard et al., 2003). After cyst ingestion by the host, a trophozoite needs to be liberated in the intestinal lumen through a process called excystation. The initiating signal of this process is the presence of H^+ ions in gastric secretions (Bingham et al., 1979), but emergence of the trophozoite in this environment would destroy it. Further triggers to finalize the excystation, such as exposure to proteases and nutrients, are present in the small intestine (Gillin et al., 1989).

Once released in the intestinal lumen, the parasite rapidly divides and colonizes the intestine with a preference for the jejunum. At this point, trophozoites appear as pear-shaped organisms with two nuclei, four pairs of flagella and a ventral disc. The flagella permit the parasite to move through the fluids in the intestine, while the ventral disc gives it the ability to attach to the intestinal wall (Adam, 2001).

Since trophozoites are unable to survive outside of the intestinal lumen, completion of the life cycle and transmission to the next host requires the formation of cysts through a process called encystation. High concentrations of bile salts, fatty acids and a slightly alkaline pH have been identified through *in vitro* studies as possible triggers of this process (Gillin et al., 1988). During encystation, proteins are produced internally and secreted to form the important cyst wall while a severe remodeling of the cytoskeleton takes place to internalize the flagella and the ventral disc (Elmendorf et al., 2003; Palm et al., 2005). The end product is a rounded cyst with four nuclei that has no mechanism to attach to the intestine and will be shed in the faecal material.

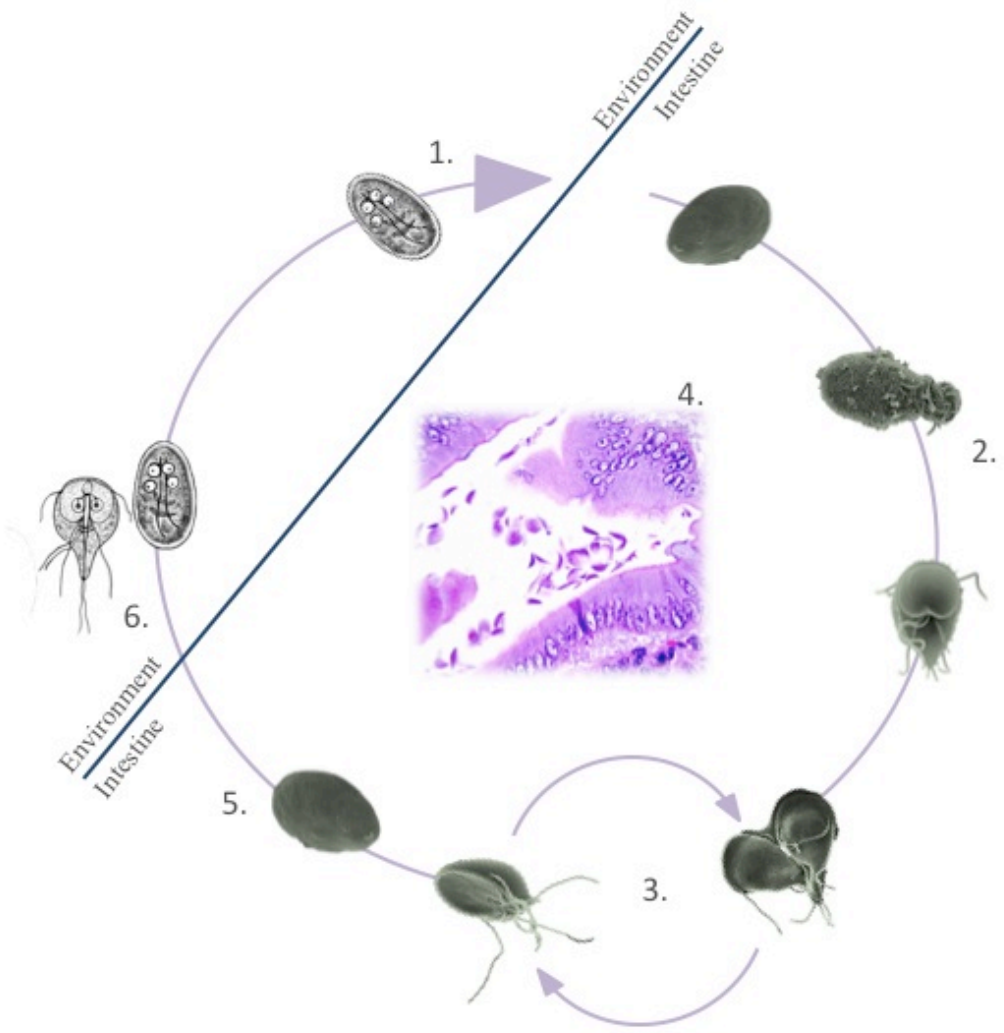

Fig 1.2. Life cycle of *Giardia*.

The life cycle of *Giardia* commences by ingestion of infectious cysts by the host (1). Once the small intestine is reached, excystation takes place and trophozoites are liberated (2). Trophozoites divide by binary fission (3) and colonize the small intestine (4). High concentrations of bile salts, fatty acids and a slightly alkaline pH may trigger a trophozoite to form a cyst (encystation) (5). The cysts and to a lesser extent trophozoites are excreted via the faeces (6). Only the cysts survive in the environment and are immediately infectious upon excretion and ready for transmission to the next host.

Giardia possesses certain key characteristics that greatly influence its epidemiology and transmission. A first important element is the immediate infectiveness of cysts. As soon as excretion occurs, they are ready to be ingested and to successfully infect a new host. In addition, only a low number of cysts is needed to establish infection: a dose as little as 10-100 cysts already proved to be sufficient in human experiments (Rendtorff, 1954; Thompson and Monis, 2004). The combination of the low infection dose with the large numbers of cysts excreted in the faeces explains the rapid spreading of *Giardia* between hosts. In ruminants, cyst excretions as high as 10^6 cysts per gram faeces were found (O'Handley and Olson, 2006) indicating that infection pressure can increase immensely in a very short period of time.

Giardia cysts remain infectious for months upon excretion depending on the environmental matrix and the surrounding temperature. A few examples are the survival of *Giardia* cysts in water of 12 weeks, 6 weeks in hard soil (Olson et al., 2004), 18 days in human faeces (Deng and Cliver, 1992) and 7 days in cattle slurry (Olson et al., 1999; Grit et al., 2012). All in all, the resistance in the environment ensures a steady build up of the environmental contamination.

1.3. Prevalence of *Giardia duodenalis*

Occurring throughout tropical and temperate regions worldwide, *G. duodenalis* can be considered as the most commonly found intestinal pathogenic protozoon in humans, with an estimated 280 million new infections every year (Lane and Lloyd, 2002). In the industrialized world, prevalence rates vary from 2 to 7% (Fletcher et al., 2012) and infections are often associated with international mobility (Ekdahl and Andersson, 2005). This was shown in a study on travel- and migration-associated illnesses, where *Giardia* was revealed as the most common parasitological cause of gastro-intestinal complaints (Field et al., 2010). In developing countries, prevalence rates vary from 20 to 30% and infections are linked to poor sanitary conditions, overcrowding and poor water quality (Younas et al., 2008). Human infections are associated with *G. duodenalis* assemblage A and B, with different studies giving a varying distribution of these assemblages,

sometimes even within a country. Worldwide, assemblage B seems to be slightly more common than assemblage A (Feng and Xiao, 2011).

The presence of *G. duodenalis* is however not limited to humans. A broad range of animals, including both companion and farm animals, are often infected with host specific and/or in several cases zoonotic *Giardia* assemblages. Prevalence rates in cats and dogs range from <1 to 45% in sheltered dogs (Upjohn et al., 2010) and 4 to 11% in cats (Olson et al., 2010). The host-specific assemblages C and D are the most regularly discovered assemblages in dogs, although assemblage A was also found at fairly high rates. In cats, both assemblage A and F can cause infection, with a more frequent occurrence of assemblage F (Feng and Xiao, 2011). Regarding production animals, worldwide prevalence data are especially available for ruminants and pigs. A study in Denmark on pigs showed a prevalence rate of 17.3% (Maddox-Hyttel et al., 2006), while an Australian study found a prevalence of 31.1% (Armson et al., 2009), predominantly of assemblage E and to a lesser extent also A (Feng and Xiao, 2011). In sheep and goats, infection rates vary from 1.5% to 55.6% and 12.3 to 42.2% respectively. These animals are mostly infected with assemblages E and A (Feng and Xiao, 2011). In cattle, prevalence rates range from 9 to 73% on an animal level, with the highest prevalence found in 1 to 6 month old animals. Looking at prevalence numbers on a farm level, percentages of 45% to 100% are mentioned in literature (Geurden et al., 2008). On a positive farm, the cumulative incidence is 100%, *i.e.* every animal will get infected on that farm once *Giardia* is present in one of them (Geurden et al., 2010). When looking at the genetic data, the livestock specific assemblage E is the dominating genotype in cattle in Europe, Australia and North America. However, in these areas assemblage A has been found as well, making it the most common zoonotic genotype. Only a small percentage of cattle have tested positive for assemblage B. New-Zealand appears to have a different distribution of assemblages compared to other areas of the world. Here, assemblage A and B are most common, whereas assemblage E is nearly absent in calves (Feng and Xiao, 2011).

1.4. Zoonotic potential of G. duodenalis

As described above, animals can harbor the same assemblages as those occurring in humans, thus hinting a zoonotic potential for *G. duodenalis*. In addition, infectivity studies with cysts isolated from both animals and humans have shown that zoonotic transmission is indeed possible (Monis and Thompson, 2003). On the other hand, when looking at the frequencies at which these zoonotic assemblages appear, they seem to be outcompeted by the host-specific assemblages E, C, D and F of the different production and companion animals.

Only a few epidemiological studies have examined the real zoonotic risk of *Giardia* infections in animals. Work done in New Zealand and the United Kingdom did not identify contact with pets or exposure to dogs or cats as a risk factor for *Giardia* infections in humans. An increased infection risk was however found for people working closely with farm animals such as farmers (Hoque et al., 2002; Hoque et al., 2003; Stuart et al., 2003).

Assemblage A is the most commonly appearing zoonotic assemblage in different animal species and has thus raised the most interest. This assemblage has been divided in sub-assemblages AI, AII and AIII, with humans being mostly infected by AII and animals with AI (Feng and Xiao, 2011). Although still not occurring often, the same subgroups are from time to time found in animals and humans. In a study done on cattle (Geurden et al., 2008), the same subgroup AII was detected in calves as was earlier described in humans. It should be noted that finding the same genotypes in humans as in animals does not warrant zoonotic transmission. Only dynamic epidemiological studies on humans and animals living in close proximity (such as the same household) could give conclusive information on the zoonotic potential of *G. duodenalis*.

1.5. Clinical signs and pathology

After its discovery, *Giardia* raised questions and debate regarding its pathogenic potential. Interestingly, its discoverer Van Leeuwenhoek did already make an association between *Giardia* and his own loose stool (Dobell, 1920) while during the First World War, soldiers with diarrhea often had *Giardia* in their faeces that gave similar symptoms

when administered to laboratory animals. It was only from 1920 onwards that important influential researchers such as Miller and Dobell supported the idea of *Giardia* as a disease causing agent (Cox, 2002).

In September 2004, giardiosis joined the WHO's Neglected Diseases Initiative. This initiative consisted of a variety of parasitic, viral and bacterial diseases that mostly occur in developing countries and for which poverty influences the outcome of the disease (Savioli et al., 2006). In addition, the pathogens included in the initiative all represent an increasing global burden and prevent infected individuals from reaching their full potential.

A *Giardia* infection in humans can occur in different forms. On the one hand, asymptomatic carriers harbor infections that go by unnoticed, while in others, *Giardia* can cause acute or even chronic disease. Symptomatic disease usually occurs after a prepatent period of 3 to 20 days (Farthing, 1996). The most common symptom associated with giardiosis is diarrhea, followed closely by weight loss. Others include epigastric pain, flatulence, nausea, vomiting and steatorrhea (Eckmann, 2003; Robertson et al., 2010; Thompson et al., 1993; Troeger et al., 2007). Although infections can be self-limiting, a proportion of patients without apparent immunodeficiencies develop chronic disease (Nash et al., 1987; Rendtorff, 1954). In these cases a persistent diarrhea is present, often resulting in profound weight loss. Especially infants and young children are at risk of developing clinical giardiosis and chronic infections can lead to malnutrition, growth impairment and even poor cognitive development (Berkman et al., 2002; Fraser et al., 2000). In a study done by Muhsen and Levine (2012), a systematic literature review and meta-analysis suggested an important role for *Giardia* in persistent diarrhea in children in nonindustrialized settings, but not in acute diarrhea.

Why exactly there is such great discrepancy in the way infected hosts respond is still unclear. Numerous attempts have been made to link these differences to the different *Giardia* assemblages that can infect humans. However, the outcomes of these studies are inconclusive. While in several studies patients infected by assemblage A appeared more likely to show clinical signs and assemblage B was associated with asymptomatic cases (Perez Cordon et al., 2008; Read et al., 2002; Sahagun et al., 2008), other studies revealed the opposite (Al-Mohammed, 2011; Mahdy et al., 2009) with assemblage B causing more

persistent diarrhea (Homan and Mank, 2001; Muhsen and Levine, 2012). Besides assemblage related parasite factors, the appearance of a new genotype in an area where a different genotype was endemic, the possible synergistic effect of different assemblages in a single host, infection pressure and multiple parasitic factors (such as rate of multiplication, ability to evade immunity and others) have all been considered as possible causes of the variability in host response (Robertson et al., 2010; Tungtrongchitr et al., 2010).

In contrast to the situation in humans, *Giardia* infections in ruminants typically lead to a chronic infection with potential intermittent symptoms in all infected individuals. In cattle, infections lead to cyst excretions that can last well over 100 days in both dairy and beef cattle (O'Handley and Olson, 2006). This type of chronicity has also been documented in goats (Koudela and Vitovec, 1998) and sheep (Sweeny et al., 2011). An infection with *Giardia* in these hosts species can cause ill thrift and diarrhea that is unresponsive to antibiotics or coccidiostatics and that is pasty to fluid with a mucoid appearance. The potential impact of such infection on production is of particular interest. Experimental *Giardia* infections in goat kids and lambs resulted in a decreased feed efficiency with decreased weight gain and lowered carcass weight (Aloisio et al., 2006; Koudela and Vitovec, 1998; Olson et al., 1995). Whether a difference in clinical outcome occurs between different assemblages in ruminants is not known.

Although often remaining without clinical symptoms, *Giardia* infection in dogs and cats can present itself with a soft watery diarrhea or steatorrhea and an abdominal discomfort or pain. In immunocompetent animals, these symptoms are usually self-limiting (Epe et al. , 2010; Tangtrongsup and Scorza, 2010).

As is the case with clinical symptoms, the pathological abnormalities caused by *Giardia* in the intestine can also vary substantially between infected individuals. A summary of the observations previously published is shown in Table 1.2. A study performed on duodenal biopsies of infected human patients could not find any association between the presence of *Giardia* and histological changes (Oberhuber and Stolte, 1990). Work done on biopsies of 567 infected people revealed a normal light microscopic appearance of the duodenal mucosa in 96.3% of the cases, with the remaining 3.7 percent showing only mild villous shortening and mild inflammation of the lamina propria

(Oberhuber et al., 1997). In ten children with chronic diarrhea caused by *Giardia*, three had completely normal jejunal tissue, five children showed mild to moderate morphological abnormalities while two showed more severe pathologies (Judd et al., 1983). Troeger et al. (2007) investigated duodenal biopsies of 13 patients with chronic giardiosis. An altered mucosal architecture was seen with a reduction of villous surface area by 50% when comparing tissue of the infected patients to the control tissue. In addition, an epithelial barrier dysfunction was detected, likely caused by increased apoptosis of enterocytes as well as a reduced expression of the tight junctional protein claudin 1. In addition, a reduced absorption of Na-glucose and an increased anion secretion were also detected.

In ruminants, a moderate villous atrophy and inflammatory infiltration has been seen in the small intestine of goat kids infected with *Giardia* in samples taken on day 21 and 28 *post infection* (Koudela and Vitovec, 1998). Work done in calves found villus atrophy and increased numbers of intraepithelial lymphocytes after less then four weeks of infection (Ruest et al., 1997), while treatment with fenbendazole and the subsequent clearance of the parasite increased the brush border surface area and intestinal disaccharidase activity in previously infected animals (O'Handley et al., 2001). In contrast to these findings, Barigye et al. (2008) described the absence of histological changes in the intestine of four out of five calves infected with *G. duodenalis* assemblage E.

In addition to the above mentioned *in vivo* studies in natural hosts, a large number of *in vitro*- and animal model studies have been performed in an attempt to unravel the underlying mechanisms in the pathogenesis of *Giardia*, focusing on the epithelial barrier malfunction in particular. *In vitro* exposure of human intestinal cell lines to *G. duodenalis* trophozoites resulted in increased enterocyte apoptosis rates in a caspase-3 dependent manner (Chin et al., 2002; Panaro et al., 2007). Similar, microarray analysis showed an up-regulated expression of genes associated with apoptosis in human Caco-2 intestinal epithelial cells after exposure to *G. duodenalis* (Roxstrom-Lindquist et al., 2005). Besides apoptosis, the co-culture of trophozoites with human small intestinal cell lines affected important junctional and cytoskeletal elements such as zonula occludens-1, α-actinin and cellular F-actin (Buret et al., 2002; Scott et al., 2002; Teoh et al., 2000). Work done in a

G. muris-mouse infection model showed that, in response to the increased permeability of the epithelium, T cells, and more specifically $CD8^+$ T cells, are activated and cause brush border microvilli shortening and the subsequent reduction of the total intestinal absorptive area (Scott et al., 2000). The loss of microvilli coincided with a deficiency in a variety of brush border enzymes such as lipase, disaccharidase, maltase and sucrase as was shown in studies done on laboratory animals (Buret et al., 1991; Cevallos et al., 1995; Gillon et al., 1982). Brush border abnormalities additionally cause an impaired absorption of water, glucose and electrolytes in infected rodents, creating an osmotic gradient that pulls water into the intestinal lumen (Cevallos et al., 1995; Scott et al., 2000; Scott et al., 2004). Another diarrhea-inducing characteristic of a *Giardia* infection is the increased intestinal transit. Research revealed that *Giardia* influences transit by inducing mast cell degranulation that in its turn causes an enhanced contraction of the smooth muscles in the intestinal wall in *G. duodenalis* infected mice (Li et al., 2007). These contractions are both responsible for abdominal cramps and for reducing the time available for water reabsorption and nutrient uptake in the intestine.

Table 1.2. *Giardia duodenalis* pathogenesis and pathology in natural hosts

Host	Individuals analyzed (*n*)	Infection & Assemblage	Pathology	References
Human	567 patients	Natural	96.3% of patients normal mucosa, in 3.7% of patients, mild shortening of villi	(Oberhuber and Stolte, 1997)
	10 children	Natural with chronic diarrhea Assemblage unknown	*n* = 5 mild, *n* = 2 severe, *n*=3 no abnormalities	(Judd et al., 1983)
	13 patients	Chronic giardiosis Assemblage unknown	All subjects: reduction in villous surface area, reduced expression of CLDN1, intestinal malabsorption and increased anion secretion	(Troeger et al., 2007)
Goat	8 goat kids	Dose: $3x10^6$ *Giardia* cysts Assemblage unknown	Moderate atrophy on day 21 and 28 *pi*	(Koudela and Vitovec, 1998)
Cattle	10 calves	4 weeks of natural infection Assemblage unknown	All infected calves villus atrophy and increased IEL	(Ruest et al., 1997)
	6 calves in each group	10^5 cyst, after 10 days: fenbendazole treatment Assemblage unknown	Higher microvillus surface area and increased disaccharidase activity in treated group, no difference in villus height between treated and non-treated group.	(O'Handley et al., 2001)
	5 calves	Natural infections Assemblage E	Changes could not be definitively attributed to *Giardia*	(Barigye et al., 2008)
	5 calves	1.5-5.1 x 10^6 cysts "bovine isolate"	Intact microvilli on *Giardia* attachment sites	(Taminelli et al., 1989)

1.6. Anti-*Giardia* immune response

The immune development process can vary substantially between infected individuals, from a fast elimination to a chronic disease, depending on the host species. In the following paragraph a summary will be made of the data available concerning immune responses against and induced by *Giardia*, more specifically in humans, ruminants and rodents. In addition, a summary of data collected during *in vivo* experiments is made in Table 1.3.

1.6.1. Humans

The information on cellular immune responses and cytokine production during *Giardia* infection in humans is still very limited. Analysis on sera of infected patients showed elevated levels of IL-5, IL-6 and IFN-γ (Matowicka-Karna et al., 2009). In a study done by Ebert (1999), peripheral blood and intestinal lymphocytes from naïve individuals demonstrated proliferation of $CD4^+$ T cells and secretion of IFN-γ in response to *Giardia* trophozoites. In a preliminary study done by Gottstein et al. (1991) and work done by Hanevik et al. (2011), a role was seen for $CD4^+$ T cells in the memory response after human giardiosis. Analysis of antibody production by B cells showed the generation of parasite specific IgA, IgM and IgG's that were present in both serum and mucosal secretions, such as saliva and breast milk, in reaction to infection (Faubert et al., 2000). The importance of antibodies in immune development was further hypothesized based on higher prevalences of chronic *Giardia* infections in patients with common variable immunodeficiency and x-linked agammaglobulinemia compared to healthy patients. However, since additional defects in the immune system exist in both these syndromes, in addition to the inability to produce antibodies, further research is needed to fully understand the role of antibodies in the immune response (Faubert, 2000).

1.6.2. Ruminants

Research on the immune response to *Giardia* in ruminants has mainly focussed on the humoral responses. In a study on calves, O'Handley et al. (2003) showed that parasite-specific antibody titers in serum of infected animals did not

increase during a four-month period. Based on this observation, the authors concluded that calves do not mount a significant humoral immune response against *G. duodenalis*, likely due to the extracellular location of the parasite or to the sequential expression of distinct variant surface proteins (VSP's). Importantly, the cyst excretions at the end of the 4 months period were not decreased compared to the start of the trial, suggesting that the animals did not yet acquire immunity. Similar results were also seen in a study on lambs which were followed during 11 weeks of infection. Analysis of parasite-specific antibody titers in serum showed no changes in IgM levels and only a small increase for IgG and IgA at week 5, 9 and 11 *post infection*, prompting the authors to conclude that the lack of a clear immune response was responsible for the chronicity of infection (Yanke et al., 1998). More recently, the kinetics of the immune response in infected cattle were monitored until the animals showed immune protection against re-infection, which occurred after approximately 3 months of infection (Grit et al., 2014). The first signs of an adaptive immune response only became visible after approximately 9 weeks of infection, as characterized by a significant proliferation of IL-17A producing $CD4^+$ αβ T cells following *in vitro* restimulation of peripheral blood mononuclear cells (PBMC) with *G. duodenalis* antigens. From 11 weeks post-infection onwards, a parasite-specific IgG1 and IgA response was detectable.

1.6.3. Rodents

Although *G. duodenalis* is not a natural parasite species of rodents, both gerbils and mice have been used extensively to investigate *Giardia*-host interactions. Gerbils can be infected orally with *G. duodenalis* cysts or trophozoites. This results in an infection with cysts detectable in the faeces of the animals for around 3 to 4 weeks. The trophozoites are eliminated from the intestine around day 30 *pi* (Belosevic et al., 1983). The observations made in gerbils following a *G. duodenalis* infection are summarized in Table 1.3. In several studies, an increased production of mucus or an increase in the number of goblet cells counted in the intestine were detected in the presence of *Giardia* (Araujo et al., 2008; Ventura et al., 2013). Around 21 days *pi*, higher mast cell counts could be detected in the intestine (Hardin et al., 1997). Infection also induces the production and secretion of intestinal IgA after about a week of infection. In addition, serum antibodies were observed after 3 weeks

(Amorim et al., 2010). In the abovementioned studies, the *G. duodenalis* isolates that have been used are all of assemblage A (human isolate). It was determined by Bénéré et al. (2010) that infection can also be established using assemblage B from human origin and E obtained from cattle.

In contrast to gerbils, the infection of mice with *G. duodenalis* is somewhat more difficult. Trophozoites of the human isolate GS(M) (assemblage B) need to be administrated through oral gavage in order to establish a patent infection. The infection can subsequently be divided into three phases: the establishment phase with the presence of large amounts of trophozoites in the intestine followed by the second phase, after approximately 13 days, during which the trophozoite numbers start to drop and finally the last phase characterized by cure or chronic infection depending on the mouse strain used (Byrd et al., 1994). The observations made in mice following a *G. duodenalis* challenge are summarized in Table 1.3. T-cell dependent mechanisms seem to be essential in the control of giardiosis in mice, as shown by the development of chronic infections in *scid* mice, mice treated with anti-CD4 and mice lacking the T cell receptor gene (Singer and Nash, 2000). Mice deficient in either TNF-α or IL-6 were also more susceptible to *G. duodenalis* infection compared to wild-type control mice, as shown by the delayed parasite elimination (Zhou et al., 2003; Singer and Kamda, 2009). In contrast, IL-4 or IFN-γ deficient mice were still able to control a *Giardia* infection similar as wild-type mice (Singer and Nash, 2000). Comparable to what was seen in gerbils, a possible effector mechanism in the response to *G. duodenalis* in mice is the recruitment and activation of mucosal mast cells. The involvement of mast cells was supported by the fact that mast cell-deficient c-kit$^{w/wv}$ mice, who can not mount a mast cell response, were unable to eliminate a *G. duodenalis* infection in the normal timespan (Li et al., 2004). The role of antibodies and B cells in the clearance of a *G. duodenalis* infection in mice is still somewhat unclear. Whereas mice genetically deficient for IgA were unable to eradicate infection (Langford et al., 2002), mice lacking the polymeric immunoglobulin receptor (pIgR), responsible for transport of IgA and IgM to the intestinal lumen, had no problem eradicating a *G. duodenalis* infection (Davids et al., 2006). However, it should be stated that these mice likely still had a low level of IgA present in the intestinal lumen. Concerning B cells, although Li et al. (2004) showed that infection of mice deficient in B cells resulted in a chronic low-level infection, a study done by Singer and Nash (2000) indicated no difference in parasite clearance in *G. duodenalis*

infected B cell deficient mice compared to wild type controls. Finally, infection experiments in mice lacking inducible nitric oxide synthase (iNOS), necessary for NO production, or mice that were unable to produce active α-defensins were still able to control the infection (Aley et al., 1994; Eckmann, 2003), suggesting that these factors are not involved in the protective immune response.

An alternative way to unravel the *Giardia*-host interplay is to use the rodent-specific species *G. muris* to infect mice. The life cycle and infection process of this parasite is basically identical to that of *G. duodenalis* in natural hosts, with the only difference that most mice strains are able to clear a *G. muris* infection after 3 to 6 weeks of contact with the parasite. Regarding the immune response, earlier work identified CD4+ T cells as important elements in parasite clearance, shown by the development of prolonged infections in nude mice (Stevens et al., 1978) and mice depleted of T helper cells (Heyworth et al., 1987). Mesenteric lymph node derived T cells from *G. muris* infected mice that were stimulated with ConA secreted both IFN-γ and IL-5 in resistant mice strains and only IL-5 in susceptible strains (Venkatesan et al., 1996). Con A stimulation of mucosal T_H-cells resulted in higher amounts of IL-4 and IFN-γ secretion in infected mice compared to uninfected controls (Djamiatun and Faubert, 1998). In addition, anti-IFNγ treatment resulted in higher parasite numbers in mice with a C57Bl/6 background, but not BALB/c (Venkatesan et al., 1996). Several studies have also focussed on the role of B cells and antibody production in the elimination of a *G. muris* infection. Concerning B-cells, a central role for these cells was shown by the inability of X-linked immunodeficient mice (*xid*) (Skea and Underdown, 1991) and B cell KO mice (Snider et al., 1988) to clear an infection. However, it should be noted that in a study done by Singer and Nash (2000), no difference in parasite clearance could be seen when comparing B cell deficient to wild-type mice, a similar result as obtained for *G. duodenalis* in mice (see above). In addition, although *xid* mice are unable to eliminate a primary infection, they are resistant to a secondary challenge infection (Skea and Underdown, 1991). Looking more closely at antibodies, the production and secretion in the gut lumen of IgG and IgA in particular seems to be a key factor in the clearance of a *G. muris* infection (Belosevic et al., 1994; Heyworth, 1986). In contrast to *G. duodenalis* infected pIgR-deficient mice, loss of this receptor severely affected the ability of mice to clear a *G. muris* infection (Davids et al., 2006), hinting that *G. muris* might be more resistant to certain immune responses than *G. duodenalis.*

Table 1.3. Immune responses against *Giardia* spp.

	Human	**Ruminant**	**Rodent**	
	G. duodenalis	*G. duodenalis*	*G. duodenalis*	*G. muris*
Innate immunity				
Goblet cells, mucin production			Increased in infected gerbils (Araujo, 2008; Ventura, 2013)	
T cells & cytokines				
T cells		Proliferation IL-17 producing $CD4^+$ γδ T cells after *in vitro* stimulation with trophozoites (cow) (Grit et al., 2014)	Chronic infection in *scid*, anti-CD4 treated & T cell receptor deficient mice (Singer et al., 2000)	Prolonged infections in CD4 depleted mice (Heyworth et al., 1987)
TNF-α			TNF deficient mice show delayed parasite elimination (Zhou et al., 2003)	
IL-5	Elevated in sera of infected patients (Ebert et al., 1999)			
IL-6	Elevated in sera of infected patients (Ebert et al., 1999)		IL-6 deficient mice show delayed parasite elimination (Singer et al., 2009)	
IFN-γ	Elevated in sera of infected patients (Ebert et al., 1999)			Higher parasite numbers in anti-IFN-γ treated mouse strains (Venkatesen et al., 1996)
B cells & antibodies				
*Parasite-specific IgA, IgM and IgG'*s	Found in sera, mucosal secretions, milk and saliva (Faubert et al., 2000))	Detectable in serum from 11 weeks p.i. onwards (Grit et al., 2014)	Production and secretion of IgA starting 1 week p.i. (gerbil) (Amorim et al., 2010) IgA deficient mice are unable to clear infection (Langford et al., 2002)	Production of IgA important for parasite clearance in mice (Belosevic et al., 1994)
B cells			Contradicting results for B cell deficiency in mice (Li et al., 2004; Singer et al., 2000)	Contradicting results in mice (Skea et al., 1991; Singer et al., 2000)
Effector mechanism				
Mast cells			More in infected gerbils (Hardin et al., 1997)	

1.6.4. in vitro assays

In addition to the *in vivo* experiments summarized above, a large number of papers have been published on the use of *in vitro* assays to investigate particular aspects of the host-*Giardia* relationship.

First, the role of mucins was investigated by looking at their effect on trophozoite attachment to an artificial surface. Whereas Roskens and Erlandsen (2002) described an inhibition of attachment in the presence of mucins purified from bovine submaxillary glands and porcine stomachs, an increased attachment to glass was actually seen in the presence of human intestinal mucins by Zenian and Gillin (1985) and Gault et al. (1987) reported that *in vitro* co-culture of trophozoites with human mucus even stimulated giardial growth.

Several studies have also used *in vitro* assays to analyse the role of dendritic cells in the initiation of the adaptive immune response against *Giardia*. It was shown by Kamda and Singer (2009) that *Giardia* trophozoites and different protein fractions of *Giardia* inhibited murine bone-marrow derived DCs to elicit a Th1 inflammatory pathway, with decreased IL-12 and increased IL-10 production. Obendorf et al. (2013) reported increased expression of CD25, CD83, and CD86, and secretion of IL-12, IL-23 and IL-10 by human dendritic cells incubated in the presence of Toll-like receptor 2 ligands and *G. duodenalis*. More recently, it was shown that incubation with *G. duodenalis* trophozoites stimulated partial maturation of bovine dendritic cells associated with altered cytokine secretion and induction of T-cell proliferation (Grit et al., 2014). So in contrast to the study of Kamda and Singer (2009), no regulatory or inhibitory effect on dendritic cells was found in cattle.

Finally, several *in vitro* studies have also been performed to identify possible effector mechanisms in the protection against *Giardia*. The addition of a NO releasing compound to a trophozoite culture was shown to have a cytostatic effect on the parasite, yet without achieving cytotoxicity (Eckmann et al., 2000). However, as previously mentioned, mice lacking inducible nitric oxide synthase (iNOS), were still able to control the infection (Aley et al., 1994). It is therefore questionable whether NO actually has a role in the protection against *Giardia in vivo*. Additional *in vitro* assays indicated that human peripheral blood macrophages were able to adhere to and ingest *Giardia* trophozoites (Hill and Pearson, 1987). However, the low efficiency by which trophozoites are ingested (less then one parasite per macrophage) and the high

trophozoite/macrophage ratio in the intestine are not in favor of macrophages being a major player in the effector mechanism (Eckmann, 2003).

1.7. Conclusion

Although our knowledge on this omnipresent parasite has increased dramatically in the last decade, certain aspects still remain unclear. This is particularly the case for the differences that exist both between and within host species in their ability to acquire immunity. Whereas some species, such as rodents and in certain cases also humans, are able to eliminate the parasite in a matter of a few weeks, other host species such as cattle develop a chronic infection that lasts for several months. Comparative analysis of the immunological mechanisms and pathways involved in the immune development process in different host species is therefore needed to fully unravel this intriguing aspect of *Giardia* infections in mammals.

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Objectives

Objectives

Giardia duodenalis is an omnipresent parasite that infects a wide range of vertebrate hosts, including humans. After ingestion, *Giardia* can cause symptoms such as diarrhea or reside in the intestine unnoticed by the host. For a yet unknown reason, there exist discrepancies both between and within host species in their ability to acquire immunity. Whereas some species, such as rodents and in certain cases also humans, are able to eliminate the parasite in a matter of weeks, other host species such as cattle develop a chronic infection that can last for several months. A detailed analysis of the immunological mechanisms and pathways involved in the immune development process in different host species is therefore needed to fully unravel this intriguing aspect of *Giardia* infections in mammals.

Therefore, the overall objective of this PhD research project was to analyse the mechanisms and pathways involved in the intestinal immune responses in two natural *Giardia*-host infection models with different kinetics of immune development, i.e. a *G. duodenalis* infection in cattle that always has a chronic character and a *G. muris* infection in mice that is typically eliminated approximately three weeks post infection.

The specific research aims were:

1) To unravel the immunological responses in the intestine of calves infected with *G. duodenalis* during the acute phase of the infection, i.e. three weeks post infection, through a genome-wide transcriptome analysis combined with histology (results described in Chapter 2).
2) To analyze and monitor the kinetics of the protective intestinal immune response in mice following a *G. muris* infection again for 3 weeks of infection with a particular focus on cytokine transcription patterns and histology (results described in Chapters 3 & 4)

Chapter 2

Microarray analysis of the intestinal host response in *Giardia duodenalis* assemblage E infected calves

Based on Dreesen, L., Rinaldi, M., Chiers, K., Li, R., Geurden, T., Van den Broeck, W., Goddeeris, B., Vercruysse, J., Claerebout, E., Geldhof, P., 2012, Microarray analysis of the intestinal host response in Giardia duodenalis assemblage E infected calves. PLoS One 7, e40985.

2.1. Introduction

The clinical manifestation of a *G. duodenalis* infection ranges from an asymptomatic carrier state to gastro-intestinal complaints such as diarrhea, weakness, weight loss, abdominal pain and possibly nausea, vomiting, flatulence and fever. Although in many cases the infection is acute and self-limiting, a significant proportion of humans will develop a chronic infection with intermittent diarrhea and cyst excretions lasting for several weeks or even months (Rendtorff, 1954; Robertson et al., 2010). In cattle, infections will always be of a chronic nature. Finding the answer as to why exactly some infections remain chronic is hindered by our limited knowledge on the *in vivo* immune response in these animals. For now, this knowledge is limited to a number of studies in which antibody levels were measured in serum and milk (O'Handley et al., 2003; Yanke et al., 1998).

So despite the importance of the parasite in cattle, there is little information available on the intestinal host-parasite interactions in these animals. Therefore, the objective of this chapter was to investigate the intestinal response in calves following a primary *G. duodenalis* infection. The chosen approach involved the use of a microarray and quantitative real-time PCR (qRT-PCR) to obtain a gene transcription profile in infected animals combined with histological work.

2.2. Material and methods

2.2.1. Infection trial and tissue collection.

This study was conducted in accordance with the E.U. Animal Welfare Directives and VICH Guidelines for Good Clinical Practice, and ethical approval to conduct this study was obtained from the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University.

Eight male Holstein calves, two to four weeks old, were used for the trial. Prior to arrival, all animals were screened three consecutive days for the presence of *Giardia* cysts and *Cryptosporidium* oocysts in their faecal samples with the use of the commercially available MERIFLUOR *Cryptosporidium/Giardia* immuno-fluorescence assay (IFA) (Meridian diagnostics Inc., Cincinnati, OH). In addition, the

animals were checked for the presence of Bovine Viral Diarrhea antigen in their blood, while faecal samples were screened for the presence of *Eimeria spp*. oocysts (McMaster method). After confirming their negative status for all these pathogens, four randomly chosen animals were placed in a pen in which *Giardia*-excreting calves had been housed prior to this experiment. The four remaining animals were kept as negative controls in separate *G. duodenalis*-free stables. All calves in the study received the same commercial milk replacer (Spraystart-Z® from Aveve; 6L per calf/day). Water and hay were provided *ad libitum* throughout the experiment. After three weeks, the presence or absence of a *G. duodenalis* infection was confirmed by IFA in faecal samples from all calves after which the animals were euthanized.

Jejunal tissue samples for gene expression analysis and histology were taken 3 meters from the pylorus. For RNA extraction, tissue was immediately snap frozen in liquid nitrogen and stored at -80°C. The same procedure was followed for the draining mesenterial lymph nodes. For histological purposes, tissue was fixed in 10% formaldehyde in phosphate buffered saline (PBS) for 24h, followed by incubation for 1h in distilled water and storage in 70% ethanol, all at room temperature. The samples were subsequently dehydrated through a series of graded ethanol solutions followed by xylene and embedded in paraffin.

2.2.2. Molecular characterization.

Faecal samples were collected in order to genotype *G. duodenalis* present in the infected animals. The necessary DNA was extracted using the QIAamp Stool Mini Kit (Qiagen) according to the manufacturer's instructions, incorporating an initial step of three freeze–thaw cycles (freezing in liquid nitrogen for 5 min and heating at 95°C for 5 min) into the protocol to maximize cyst lysing. The eluted DNA was dissolved in 15 µl of ultra-pure water.

A nested PCR targeting the triosephosphate isomerase (*tpi*) gene was used for specific amplification of assemblage A and E, as described by Geurden et al. (Geurden et al., 2008).

2.2.3. RNA extraction.

Extraction of total RNA from tissue samples was done using TRIzol

(Invitrogen) followed by further purification with the RNeasy Mini kit (Qiagen) following the manufacturer's instructions. An on-column DNase digestion was performed using the RNase-free DNase set (Qiagen) to remove any contaminating genomic DNA. Total RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and the quality of the RNA was verified with an Experion™ Automated Electrophoresis System (Bio-Rad). For all samples, the RNA quality indicator (RQI) calculated by the Experion™ software (Bio-Rad) was >8.0, indicating high RNA integrity.

2.2.4. Microarray analysis and Ingenuity Pathway Analysis.

The RNA extracted from the jejunum of all the animals was used for microarray analysis. Double-stranded cDNA was synthesized using the Superscript™ Double-Stranded cDNA Synthesis kit (Invitrogen) followed by further concentration using the DNA Clean and Concentrator™-25 (Zymo Research) following manufacturer's instructions. Microarray (Roche Nimblegen, Inc., Madison, WI) design and hybridization procedures were performed as previously described (Li and Li, 2006). The bovine microarray consisted of 86,191 unique 60mer oligonucleotides, which represents 45,383 bovine sequences or genes. Each unique oligonucleotide was repeated four times on the microarray. After hybridization, scanning and image acquisition, data extraction from the raw images was executed using NimbleScan software (NimbleGen, Madison, WI). For each feature, relative signal intensities (log2) were generated using the robust multi-array average algorithm (Irizarry et al., 2003). Further processing of the data was done based on the quantile normalization method (Bolstad et al., 2003). The background-adjusted, normalized and log-transformed intensity values were further analyzed using MeV v4.2 (http://www.tm4.org/mev/). Raw and processed microarray data were deposited to the NCBI Gene Expression Omnibus (GEO) database (Accession# GSE35920).

The Ingenuity Pathway Analysis (IPA) software V6.0 (Ingenuity® Systems, www.ingenuity.com) was used to organize the genes regulated during the infection into networks of interacting molecules. The gene identifiers of the genes with a statistically significant change in expression ($p < 0.01$ and $p < 0.05$) and with a calculated positive or negative fold change of at least two-folds were uploaded in the software. These genes, called focus genes, were overlaid onto a global molecular

network developed from information contained in Ingenuity's Knowledge Base. Networks were then algorithmically generated based on their connectivity. Each network is assigned a score, a numerical value that ranks the networks according to how relevant they are to the genes in the uploaded dataset based on the number of focus genes and the size of the network. In addition, IPA was used for a functional analysis to identify the biological functions that were most significant to the uploaded datasets. Right tailed Fisher's exact test was used to calculate a p-value determining the probability that each biological function assigned to that dataset is due to chance alone. The same method is used to analyze the biological functions of a calculated network.

2.2.5. Quantitative Real-time PCR.

A qRT-PCR approach was used to validate the generated microarray data. The gene expression data of five upregulated and two downregulated genes were used as endogenous controls and analyzed with qRT-PCR. These genes include ATP-binding cassette sub-family G (WHITE) member 8 (ABCG8), adenosine deaminase (ADA), fatty acid synthase (FASN), peroxisome proliferator-activated receptor alpha (PPARα), peroxisome proliferator-activated receptor gamma (PPARγ), RAS guanyl releasing protein 2 (RASGRP2) and ras homolog gene family, member D (RHOD). In addition, qRT-PCR was also used to further elucidate the host-response during infection by analyzing expression of genes coding for the following cytokines: INF-γ, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-13, IL-17, TGF-β1 and TNF-α.

The qRT-PCR analyses were performed using the SYBR Green Master Mix (Applied Biosystems) on cDNA samples produced using the iScript cDNA synthesis kit following the manufacturer's instructions. Two µl of single stranded cDNA (10 ng of the input total RNA equivalent) and 500 nM of amplification primer were used in a reaction volume of 20 µl. The primer sets used to amplify the different bovine genes were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/) and are listed in the appendix.

A StepOnePlus Real-Time PCR system (Applied Biosciences) executed the amplification cycles as follows: 95°C for 20 s; 35 cycles of 95°C for 5 s; and optimal annealing temperature (Ta) for 30 s. For all genes tested, the Ta was set at 60°C, with the exception of IL-4 where the Ta was 64°C. Reaction efficiencies were measured

based on a standard dilution curve obtained by serial dilutions of pooled cDNA material from all the samples. To ensure specificity of the primers, melting curve analyses were performed at the end of the reactions, and the obtained PCR products were sequenced. In every assay, cDNA samples were analyzed in duplicate, and a non-template control was added. The obtained Ct values were transformed in relative quantities using the delta Ct method, which applies following formula: $Q=E^{(\text{min Ct-sampleCt})}$. In this formula, Q represents the relative quantity for each sample, E the amplification efficiency of the run, min Ct is the lowest Ct value among all the samples for each gene analyzed and sampleCt is the Ct for each sample in the run (http://medgen.ugent.be/~jvdesomp/genorm/geNorm_manual.pdf). These quantities were normalized against internal control genes, referred to as housekeeping genes (HKGs). The correct HKG were selected out of a panel of 6 candidate genes (beta actin (*ACTB)*, hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), ribosomal protein P0 (*RPLP0*), succinate dehydrogenase flavoprotein subunit A (*SDHA*), TATA box binding protein (TBP)-associated factor (*TAF2*), and ubiquitin-conjugating enzyme E2D2 (*UBE2D2*) based on the gene stability measure M calculated by the GeNorm (geNorm3.5) software using the developers recommendations (Andersen et al., 2004). This identified UBE202 and ACTB as the most suitable HKG for jejunal samples and RPLP0 and HPRT1 for tissue of mesenterial lymph nodes.

Statistical analysis was carried out using GraphPad Prism software. The Nonparametic Whitney U test was used to determine differences between the infected and the control group. A P-value ≤ 0.05 was considered significant.

2.2.6. Histology.

Jejunal tissue sections of 4 µm were cut and mounted on APES-coated glass slides. Immunohistochemical stainings with a polyclonal rabbit anti-human CD3 (Dakocytomatio A/S, Glosturp, Denmark), a rabbit anti-human CD-20 (Neomarkers, CA, USA), and a monoclonal mouse anti-human MAC387 (Serotec, Cergy Saint-Christophe, France) were performed to identify and count T-cells, B-cells and macrophages, respectively, as previously described by Vangeel et al. (2011) (Vangeel et al., 2012). Briefly, this included the use of the peroxidase streptavidin complex (Dakocytomatio A/S, Glosturp, Denmark), diaminobenzidin tetrahydrochloride

(DAB, Sigma-Aldrich, St. Louis, USA), and H_2O_2 followed by counterstaining with haematoxylin. Quantification was done by counting positive cells in the lamina propria of a villus and associated crypt with light microscopy (Olympus BS 61, Olympus Belgium, Aartselaar, Belgium), via a 40x objective (400x magnification). Five appropriate areas were chosen randomly on each slide and cells were expressed as number of cells per $10^5 \mu m^2$. Intraepithelial lymphocytes (IEL) were calculated by counting the number of CD3 positive lymphocytes per 100 enterocytes along the villus at 400x magnification. For this, at least 500 enterocytes were counted per sample.

To quantify the number of eosinophils and mast cells in the jejunal mucosa, tissue sections were stained with haematoxylin-eosin (HE) and Giemsa, respectively. For each cell type, 10 non-overlapping high power fields (HPF) (400x) were checked and numbers were summed up to give a total cell count per 10HPF.

To quantify apoptosis, apoptotic cells were counted using fluorescence microscopy (400x) on tissue slides (4µm) stained with the DeadEnd^{TM} Fluorometric TUNEL system (Promega). Staining was performed following the manufacturer's recommendations. Counterstaining of the nuclei was done using DAPI (4', 6-diamidino-2-phenylindole, dilactate; 1:1000 in PBS; Invitrogen) for 5 minutes at room temperature. Five appropriate areas were chosen randomly on each slide, and apoptotic cells were expressed as number of cells per $10^5 \mu m^2$.

Finally, the length of the villi and depth of the crypts in the jejunum were measured by analyzing 15 villi and their corresponding crypts under a microscope using a calibrated micrometer at 200x magnification. All results were compared by the student's *t*-test using GraphPad Prism software. A P-value ≤ 0.05 was considered significant.

2.3. Results

2.3.1. Infection trial.

After 3 weeks of environmental exposure to *G. duodenalis* cysts, all four animals in the infected group tested positive for *Giardia* cysts in their faeces. Mean cyst excretion varied between 3950 and 15,000 cysts per gram (CPG) of faeces with

an average of 10,328 CPG. Genotyping of the collected cysts revealed that animals were infected with the livestock-specific *G. duodenalis* assemblage E. The control group remained negative throughout the study.

2.3.2. Transcriptomic profiles and pathway analysis.

A bovine high-density oligo microarray was used to analyze global gene expression in the small intestine of the calves. At a 95% confidence level ($P < 0.05$), 140 genes were differentially expressed after infection with a minimum two-fold change (see appendix), 57 genes were transcriptionally downregulated, and 83 genes were upregulated.

Up-or down-regulation of 7 selected genes which showed the highest fold changes according to the microarray data were subsequently verified by quantitative PCR. Consistent with the microarray results, the expression of PPARα and PPARγ, ATP-binding cassette transporter (ABCG8), adenosine deaminase (ADA), and Ras homolog gene family member D (RHOD) was upregulated in the infected animals compared to controls, while a downregulation was confirmed for Ras guanyl releasing protein 2 (RASGRP2) and fatty acid synthase (FASN) (Figure 2.1); however, the downregulation of RASGRP2 as measured by qRT-PCR was not significant.

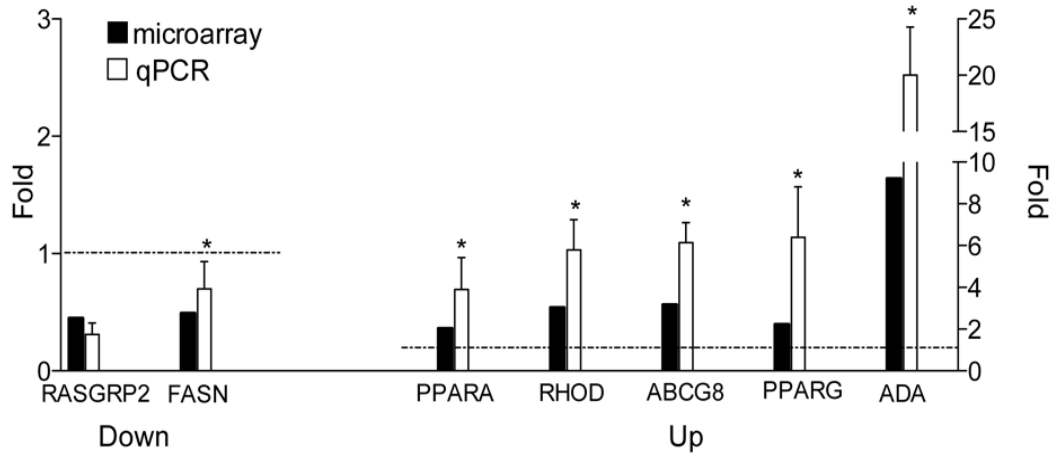


Figure 2.1. **qRT-PCR validation of selected differentially expressed genes identified by microarray.** Quantitative PCR on a panel of 7 genes was used to verify the up-or-down regulation observed by microarray analysis. Consistent with the microarray results, expression of PPARA, RHOD, ABCG8, PPARG and ADA was upregulated when comparing infected animals to controls, while a downregulation could be seen for RASGRP2 (although not significant) and FASN. (* $P < 0.05$).

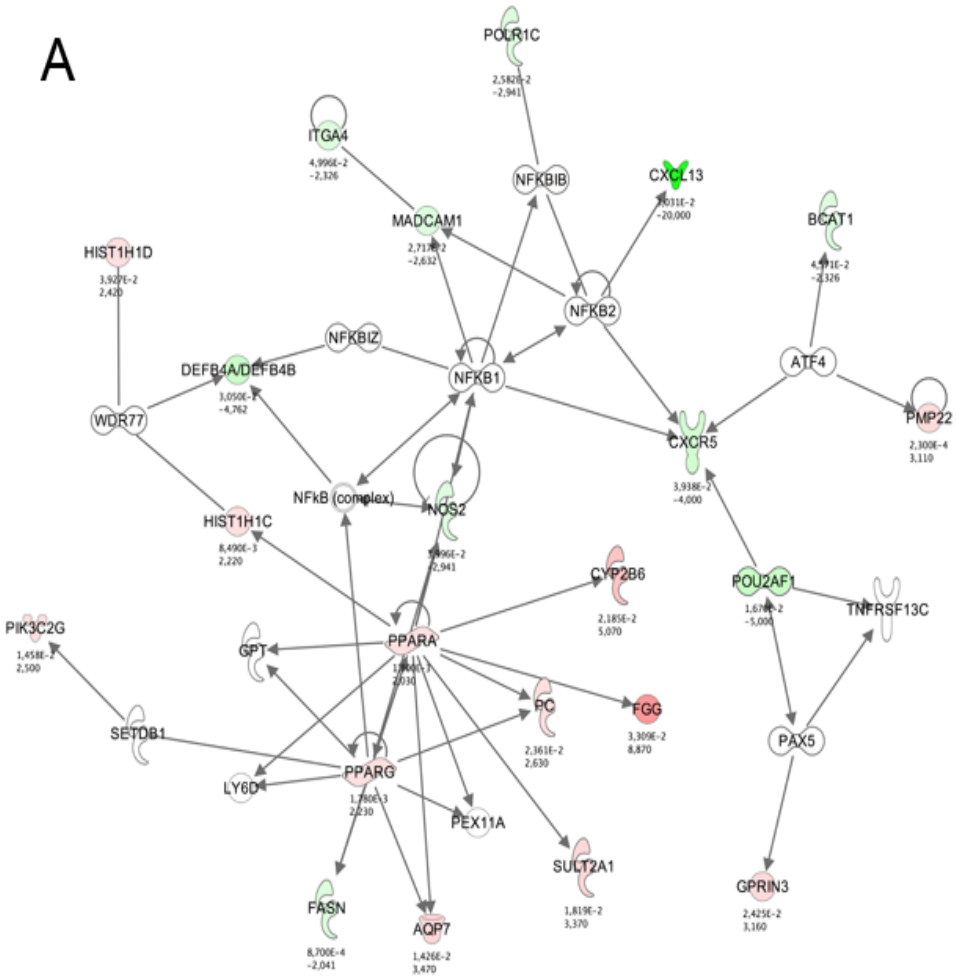
The IPA software was subsequently used to further examine the microarray dataset. When the dataset was uploaded, IPA determined the biological functions related to this dataset and gave the predicted activity of these functions. Overall, 4 main categories of functions were associated with the uploaded dataset: migration of leukocytes, inflammation, immune response, and lipid metabolism (summarized in Table 2.1.). A total of 19 genes were related with cellular migration of B-cells, T-cells, phagocytes, and granulocytes, a function for which IPA predicted decreased activity. Two other functions impacted by the infection are part of the lipid metabolism. These include a decrease in lipid quantity (10 genes involved) as well as a decreased production of eicosanoids (5 genes involved). Finally, two functions labeled as 'immune response' and 'inflammation' both had a predicted decreased activity, with involvement of respectively 19 and 11 genes. The analysis also indicated that 4 genes were involved in all of the impacted functions, i.e. ADA, NOS2 (nitric oxide synthase 2), PPARα, and PPARγ.

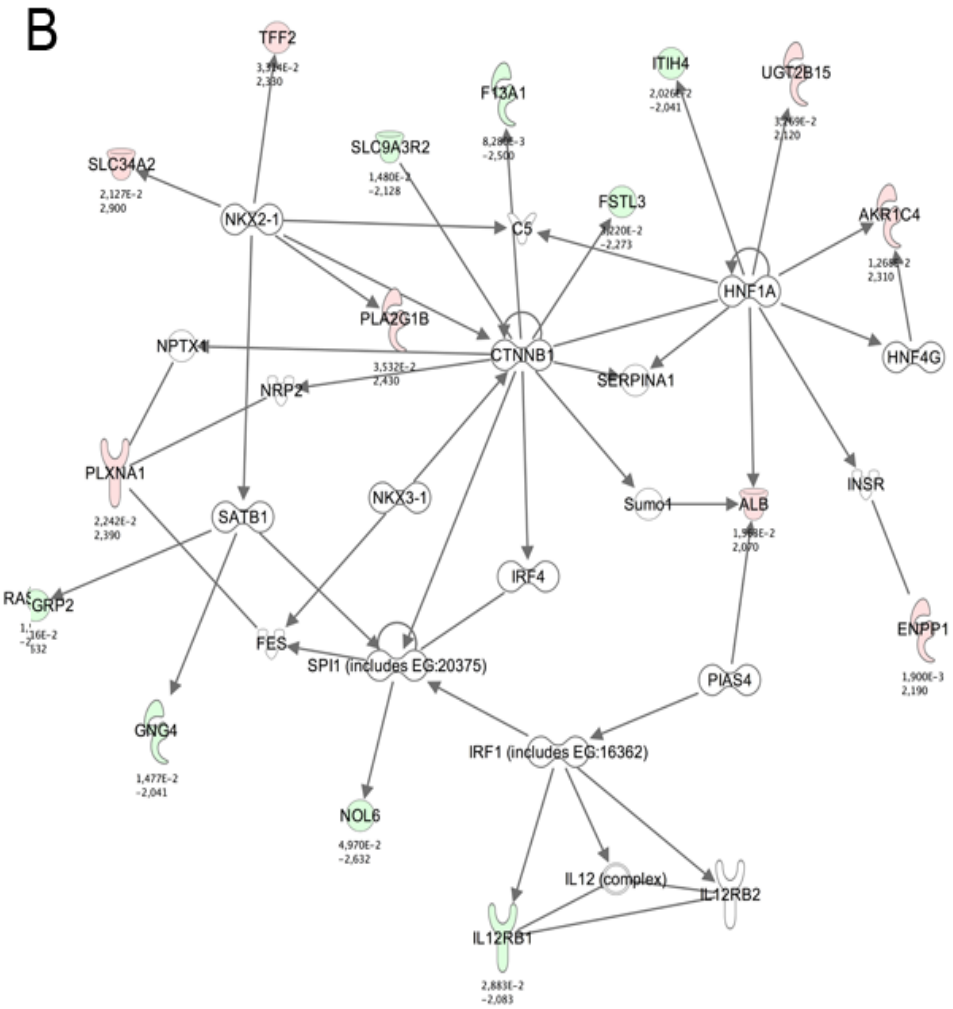
Three networks were calculated by IPA based on the uploaded $p<0.05$ dataset with a score higher than 20, implying that there is a probability of 10^{-20} that the genes in this network are solely associated by chance. The highest scoring network (score of

39) again deals with immune cell trafficking with a central role for the PPAR genes (Figure 2.2, panel A). Network 2 had a score of 25 and is associated with tissue morphology, cellular growth/proliferation, and cellular development. Finally, the network functions of the third network (score of 23) included lipid metabolism, molecular transport, and small molecule biochemistry.

Table 2.1. Impacted biological functions and associated genes during *Giardia duodenalis* infection in calves as calculated by IPA.

Function	**Prediction by IPA**	**Genes involved upregulated**	**Genes involved downregulated**
Inflammatory response	Decreased	ADA, NT5E, PPARA, PPARG, CCL14, PLA2G1B, FGG	NOS2, IL12RB1, CXCL13, DEFB4A
Immune response	Decreased	ADA, NT5E, PPARA, PPARG, PLA2G1B, CCL14, FGG, PLXNA1	HSPH1, MADCAM1, IL12RB1, CXCL13, DEFB4A, CD79B, ITGA4, CD1B, NOS2, MS4A1, POU2AF1
Migration of leukocytes	Decreased	ADA, NT5E, PPARA, PPARG, PLA2G1B, CCL14, ALB	MADCAM1, NOS2, IL12RB1, CXCL13, RASGRP2, SH2D3C, F13A1, ITGA4, CXCR5, DEFB4A, POU2AF1, MAP4K1
Quantity of lipid	Decreased	AQP7, ADA, PPARA, HPGD, PLA2G1B, ALB, PPARG, LRAT, ABCG8	NOS2
Production of eicosanoid	Decreased	HPGD, PLA2G1B	NOS2, FASN, DEFB4


A
POLR1C
ITGA4
NFKBIB
CXCL13
MADCAM1
BCAT1
HIST1H1D
NFKB2
NFKBIZ
ATF4
DEFB4A/DEFB4B
NFKB1
WDR77
PMP22
CXCR5
NFkB (complex)
NOS2
HIST1H1C
CYP2B6
POU2AF1
TNFRSF13C
PIK3C2G
GPT
PPARA
PC
FGG
SETDB1
PAX5
PPARG
LY6D
PEX11A
SULT2A1
GPRIN3
FASN
AQP7


B
TFF2
F13A1
ITIH4
UGT2B15
SLC9A3R2
SLC34A2
NKX2-1
C5
FSTL3
AKR1C4
HNF1A
PLA2G1B
NPTX1
CTNNB1
HNF4G
NRP2
SERPINA1
PLXNA1
NKX3-1
Sumo1
ALB
INSR
SATB1
IRF4
GRP2
FES
SPI1 (includes EG:20375)
ENPP1
PIAS4
GNG4
IRF1 (includes EG:16362)
NOL6
IL12 (complex)
IL12RB2
IL12RB1

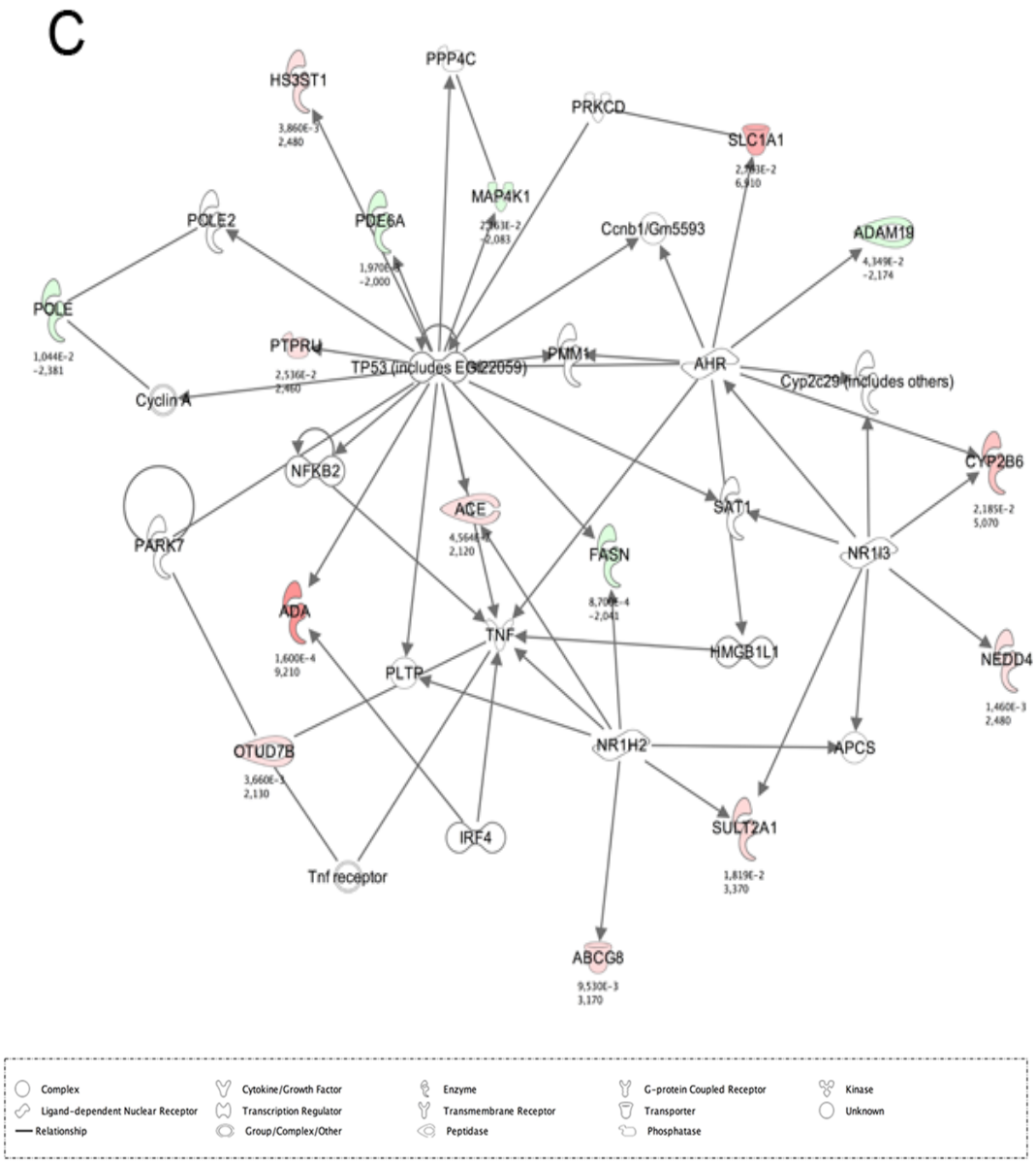


Figure 2.2. Regulatory networks impacted in the jejunum of *Giardia duodenalis* infected calves.

(A) Score of 39. The network functions include immune cell trafficking and cell movement. (B) Score of 25. Functions include tissue morphology, cellular development, cellular growth and proliferation. (C) Score of 23. Functions include lipid metabolism, molecular transport and small molecule biochemistry. Color code: red, upregulated gene; green, downregulated gene. The degree of up- or downregulation is represented by the intensity of the colors, ranging from 2-fold to 20-foldchange.

2.3.3. Analysis of the intestinal immune response.

Since the microarray data suggested a lack of inflammation, immune response and immune cell migration, the intestinal immune responses in the animals were investigated in more detail by analyzing cytokine transcription levels combined with histological analyses, such as immune cell counts, quantification of apoptosis, and villus/crypt measurements. The results of the qRT-PCRs on a selected panel of cytokines are shown in Figure 2.3 as mean fold changes in infected animals compared to the control animals. In jejunal tissue, no significant differences in transcription levels were found for IFN-γ, IL-4, IL-6, IL-8, IL-10, TGF-β1, and TNF-α. The expression of IL-13, IL-17, and IL-1β was significantly downregulated with a fold change of 0.43, 0.06, and 0.59 respectively. There was no significant alteration of cytokine expression in the mesenteric draining lymph nodes of infected animals versus those of control animals.

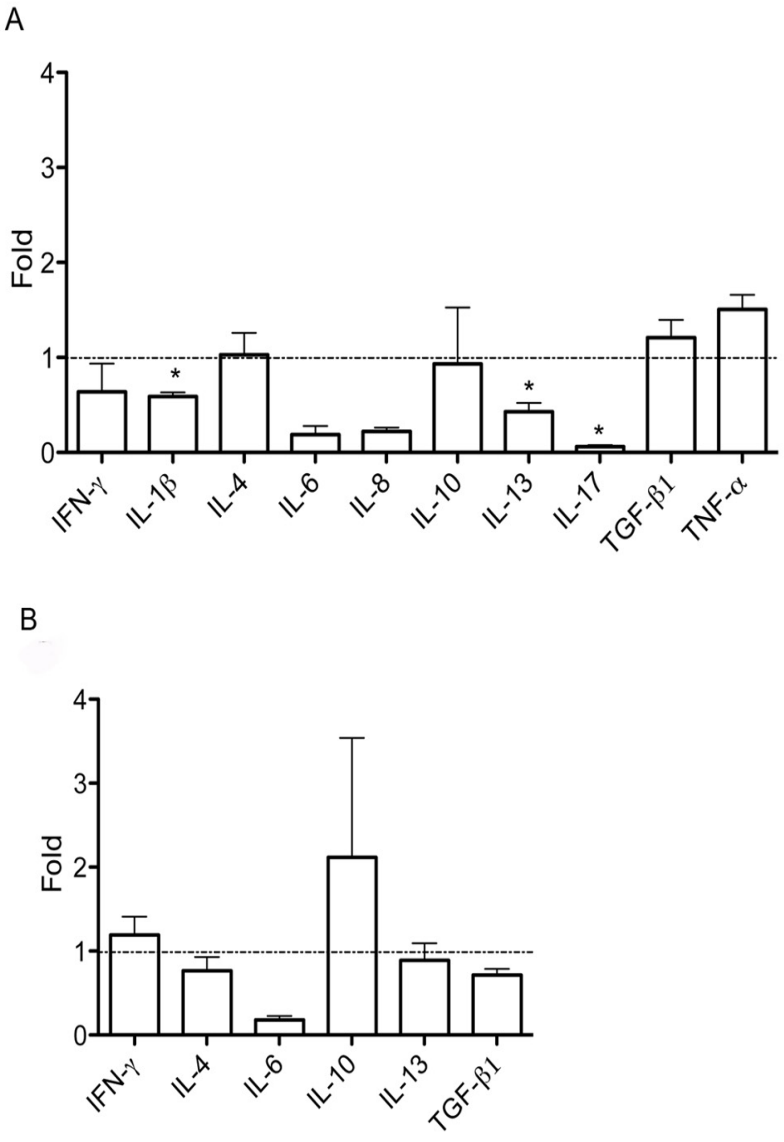


Figure 2.3. Cytokine expression profiles in jejunum and mesenterial lymph nodes of *Giardia duodenalis* infected calves. Transcription levels of the main cytokines were analyzed using quantitative PCR. Mean fold changes (n = 4) when comparing infected animals to controls are presented with SEM as error bars. In jejunal tissue (panel A), no significant differences in transcription levels were found for INF-γ, IL-4, IL-6, IL-8, IL-10, TGF-β1 and TNF-α. The transcription of IL-13, IL-17, and IL-1β was significantly downregulated. In the draining mesenteric lymph nodes (panel B), there was no significant alternation of cytokine transcription. (* $P < 0.05$).

Upon histological examination of the samples, the general morphology of the jejunal mucosa appeared to be normal without signs of inflammation. The results of the cell counts and villus/crypt measurements are summarized in Table 2.2 and 2.3. No significant differences were observed for T cells, B cells, mast cells, eosinophils, or the number of apoptotic cells between infected and control animals. Only the number of macrophages was significantly lower in infected animals (2.17 per 10^5µm) than in controls (3.17 per 10^5µm). A significant difference was also found for the villus/crypt ratio with an increased ratio in the infected animals ($p < 0.05$).

Table 2.2. Histological cell counts in jejunal tissue of control animals and *Giardia duodenalis* infected calves.

	IEL[a]	**T-cells [b] (CD3+)**	**B-cells [b] (CD20+)**	**Macrophages[b] (MAC387+)**	**Eosinophils[c]**	**Mast cells[c]**
Control	23.2 ± 4.1	240 ± 37.5	2.6 ± 1.3	3.2 ± 0.6	549 ± 295	23 ± 19
Infected	18.7 ± 3.1	217 ± 23.1	4.7 ± 2.7	2.2 ± 0.2*	348 ± 128	43 ± 40

[a] intraepithelial lymphocytes per 100 enterocytes

[b] number of cells per 10^5µm^2

[c] number of cells per 10HPF

*P ≤ 0.05 versus control group, data are mean ± SEM

Table 2.3. Villus and crypt measurements in jejunal tissue of control animals and *Giardia duodenalis* infected calves.

	Villus length (µm)		**Crypt depth (µm)**		**Villus/crypt ratio**	
	Mean ± sd	Range	Mean ± sd	Range	Mean ± sd	Range
Control	378 ± 27	313-447	237 ± 27	203-337	1.45 ± 0.11	1.26-1.68
Infected	506 ±47	385-584	253 ± 23	201-308	2.17 ± 0.15*	1.91-2.59

* P ≤ 0.05 versus control group

2.4. Discussion

In this study, gene transcription in the jejunum of uninfected and primary infected calves was analyzed using a whole genome microarray to increase our knowledge on host-parasite interactions in natural hosts, more specifically in cattle. This generated a list of functions and networks in IPA of which some can be related to suppression of inflammation and immunity. Further qRT-PCR analysis of a selected panel of cytokines revealed a trend of downregulated expression in infected animals compared to the negative controls. In addition, no immune cell recruitment was detected in the intestine of the infected calves.

Microarray analyses identified the activation of PPARα and PPARγ as the possible regulators of the observed host responses. These nuclear receptors can be expressed in epithelial and endothelial cells as well as in immune cells such as T cells, B cells, dendritic cells, and macrophages (Straus and Glass, 2007). The PPARs belong to the subfamily of the nuclear receptors, which also includes the receptors for retinoic acid, vitamin D and others. Similar as these receptors, the PPARs consist of 5 distinct regions, from which the DNA-binding domain (DBD) and the ligand-binding domain (LBD) are the most conserved. Usually PPAR activation occurs when an agonist binds the LBD. To modulate DNA transcription, the PPARs need to bind to nucleotide sequences, called PPAR response elements (PPRE), which are located in the regulatory regions of their target genes. To complete the DNA binding and interact with the PPRE, it is necessary for the PPARs to form a heterodimer with a 9-*cis*-retinoic acid receptor (RXR). Ligand binding to PPARα induces conformational changes in the PPAR-RXR complex that allow the receptors to dissociate from co-repressor complexes and associate with co-activators. These co-activators rearrange chromatin packaging so that the transcriptional machinery has access to the promoter region of the target gene (Contreras et al., 2013; Daynes and Jones, 2002; Straus and Glass, 2007). The activation of PPARs can exert an anti-inflammatory effect by transrepressing the activity of several transcription factors, such as nuclear factor-κB (NF-κB) and activator protein 1 (AP1) (Daynes and Jones, 2002). Through this transrepression, the release of pro-inflammatory cytokines (IL-6, TNF-α, IL-12, IL-17, and IL-1β) and iNOS can be inhibited, as well as the recruitment of leukocytes (Glass and Ogawa, 2006; Straus and Glass, 2007). Whether the PPAR activation actually leads to the negative modulation of the inflammatory and immune response

observed in the *Giardia* infected calves is still unclear. Further studies using PPAR agonists and antagonist are needed to provide direct evidence of the biological role of these receptors in the host response. Interestingly, the up-regulation of PPARγ was also observed in Caco-2 human intestinal epithelial cells after being incubated with *G. duodenalis* trophozoites for 18 hours (Roxstrom-Lindquist et al., 2005).

How exactly PPAR activation occurs during the *Giardia* infection in these calves is unclear. The endogenous ligands of the PPARs are various fatty acids and metabolites of fatty acid metabolism that can be produced by inflammatory responses (Daynes and Jones, 2002). The ability to induce activation of PPARs was seen in other parasite infections such as *Plasmodium spp.*, *Toxoplasma gondii,* and protozoa of the *Leishmania* genus. *Plasmodium* spp. rupture red blood cells they infect. This leads to the formation of hemozoin, in its turn causing the release of the PPARγ ligand 15-hydroxyeicosatetraenoic acid (15-HETE). *Toxoplasma gondii* induces the production of PPAR ligands by platelets, while visceral *Leishmania* infection induced PPARγ expression on residual macrophages, liver and spleen of mice (Chan et al., 2010). Sphingolipids could be the responsible activators of the PPARs during a *Giardia* infection. These lipids have shown to be possible ligands for PPARs (Tsuji et al., 2009) and are also produced *de novo* by this parasite (Zhang et al., 2010).

Besides the PPARs, other genes could be involved in the observed immune suppression, as function analysis by IPA indicated. An example is ADA, a gene coding for adenosine deaminase that is upregulated in the infected animals. This enzyme is involved in the deamination of adenosine, a purine that can exert several effects on inflammation, both pro- and anti-inflammatory (Blackburn et al., 2009). The end product of adenosine deamination is inosine, which in itself also has an anti-inflammatory effect (Hasko et al., 2004).

Furthermore, no intestinal pathologies, such as villus shortening or increased levels of apoptosis were observed in the intestines of infected animals. The available information on pathophysiological changes induced by a *Giardia* infection in humans and animals is often contradictory. While some studies report histological changes in the villi and crypts and increased levels of intraepithelial lymphocytes in infected hosts (Cotton et al., 2011; Ruest et al., 1997), others describe the lack of inflammation or other typical histological features in the majority of the patients investigated (Oberhuber et al., 1997). The reason for this discrepancy is still unclear, although some authors have suggested that assemblage-specific differences might exist. In a

study by Chin et al. (2002) (Chin et al., 2002), the ability of *G. duodenalis* to induce apoptosis in enterocytes appeared to be strain dependent. In humans, patients infected with assemblage A are more likely to present clinical signs than those infected with assemblage B, although once established infections with assemblage B seem to result in more persistent diarrhea (Homan and Mank, 2001; Read et al., 2002). Whether the general absence of pathologies or immune response in the current experiment was due to the infection with assemblage E needs to be further studied. Interestingly, Barigye et al. (2008) (Barigye et al., 2008) previously described the absence of histological changes in the intestines of four out of five calves infected with assemblage E, supporting the observations made in this study. Alternatively, the presence or absence of inflammation, histological damage and/or clinical signs may depend on the infection dose. In this experiment, calves that were introduced into a contaminated housing excreted between 3950 and 15,000 CPG. While similar infection levels are often observed in field conditions, naturally infected calves can excrete up to 10^6 CPG (Geurden et al., 2004; Xiao et al., 1996).

Apart from the apparent immunosuppressive pathway induced by the *Giardia* infection, several genes involved in lipid metabolism were also impacted, such as genes coding for enzymes involved in lipid synthesis or lipolysis (PLA2G1B, CYP3A5, HPGD, ADA), transcription factors regulating the lipid metabolism (PPARα, PPARγ), and the ABC transporter ABCG8, responsible for efflux of cholesterol from enterocytes into the intestinal lumen or as high-density lipoproteins in plasma. Theoretically, the observed transcriptional changes would lead to a decreased uptake of lipids in the intestine and a higher efflux of cholesterol in the intestinal lumen. This host response could actually be beneficial for the parasite, since several studies have previously shown that some protozoa, including *Giardia,* are unable to synthesize the majority of their lipids and cholesterol *de novo* and rely on the host's intestinal milieu for these products (Das et al., 2002). Interestingly, it has been reported that *Giardia*-infected human patients often show intestinal fat malabsorption (Katelaris et al., 1991) and significantly lower levels of total serum cholesterol compared to healthy controls (Bansal et al., 2005). Whether this is the result of a similar intestinal response in humans as observed in cattle still needs to be further examined.

In conclusion, the outcome of the current study suggests that a primary *G. duodenalis* assemblage E infection in calves results in a lack of inflammation,

immune response and immune cell migration in the infected animals. This could explain the often chronic nature of a *G. duodenalis* infection in cattle and the lack of inflammation in the intestinal tissue. Whether *G. duodenalis* infections in other host species or *G. duodenalis* assemblage A infections in calves induce a similar intestinal response is still unclear and requires further research.

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Chapter 3

The intestinal immune response in *Giardia muris* infected mice

Based on: Dreesen L., De Bosscher K., Grit G., Staels B, Lubberts E, Bauge E., Geldhof P. (2014). *Giardia muris* infection in mice is associated with a protective IL-17A response and induction of the Peroxisome Proliferator-Activated Receptor Alpha. Infection and Immunity. IAI.01536-14.

3.1. Introduction

In chapter 2, the intestinal host response in calves infected with the ruminant-specific *G. duodenalis* assemblage E was investigated. Using a bovine high-density microarray to analyse global gene transcription combined with histological work, the acute phase of the infection in cattle was marked by a lack of inflammation and immune cell infiltration.

In this chapter the objective was to gather further insights in the intestinal host response by using a murine giardiosis model where mice are infected with their natural parasite *G. muris*. The life cycle and infection process of this parasite is basically identical to that of *G. duodenalis*, with the only difference being that most mice strains are able to clear a *G. muris* infection after 3 to 6 weeks of contact with the parasite (Roberts-Thomson and Mitchell, 1978). Regarding the development of immunity against *G. muris*, earlier work already indicated the importance of CD4+ T cells (Heyworth et al., 1987; Stevens et al., 1978), B cells (Skea and Underdown, 1991; Snider et al., 1988) as well as IgG and IgA antibody production (Daniels and Belosevic, 1994; Heyworth, 1986). Work done on the mucosal inflammatory response in *G. muris* infected mice also suggested mast cells, mucus production and IFN-γ as possible elements in the host response (Venkatesan et al., 1997). However, further information regarding the type of immune response (Th1-Th2-Th17), regulatory pathways and effector mechanisms involved and, finally, the dynamics of the whole immune development process is still missing. Therefore in this chapter the cytokine transcription patterns in the intestinal tissue at different time-points following a *G. muris* infection were analyzed in BALB/C and C57Bl/6 mice, two mouse strains known to develop immunity against this parasite.

3.2. Material and methods

3.2.1. Ethical statement.

This study was conducted in accordance with the E.U. Animal Welfare Directives and VICH Guidelines for Good Clinical Practice. Ethical approval was obtained from the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University.

3.2.2. Infection studies.

In each experiment, mice were infected orally with 10^3 *G. muris* cysts suspended in 0.2 ml phosphate buffered saline. *Giardia muris* cysts were obtained from Prof Dr M. Belosevic, University of Alberta, Edmonton, Canada. The different infection trials are summarized in table 3.1.

In a first infection experiment, female BALB/c animals of 6 weeks old (Harlan laboratories) and the appropriate negative controls (all groups: *n*= 5) were used. Cyst counts were monitored daily to analyze the infection pattern and intestinal samples were taken from the control animals and the infected animals at week 1, 2 and 3 *pi* (*n* = 5 at each time point).

Infection experiment 2 consisted of infected female C57Bl/6 animals (Harlan laboratories) and the appropriate negative controls (all groups: *n*= 5). All animals were 6 weeks old at time of infection. In this trial, cyst counts were performed daily and intestinal samples were taken from the control mice and the infected animals at week 1, 2 and 3 *pi* (*n* = 5 at each time point).

For infection experiment 3, female IL-17 receptor A KO (Flierl et al., 2008) mice were used. This mouse line has been crossed back into a C57BL/6J background for over five generations. These animals (*n* = 5) were infected for 3 weeks where cyst counts were counted daily and compared to the appropriate C57Bl/6 WT controls (*n*=5).

Infection experiment 4 used infected female C57Bl/6 animals. Starting day 1 *pi*, 5 animals were necropsied daily until day 7 for intestinal samples. Every day, 2 negative controls were necropsied and pooled as one group at the end of the trial.

3.2.3. Cyst counts.

To monitor the course of a *G. muris* infection, cyst excretion in faeces was measured as described previously by Roberts-Thomson et al. (1978). In summary, faeces were collected from each mouse individually over a 2 h period and weighed. Next, the stool was homogenized with PBS and centrifuged at low speed (800g, 15min) over a 1M sucrose gradient. The obtained cysts in the top layer were further washed, resuspended in 1 ml of PBS and counted in a hemacytometer. Cyst excretion was then expressed as number of cysts per gram faeces.

Table 3.1. Summary of mice experiments

Expt nb	Objective	Strain	Age (w)	Sex	Trial duration	Number of animals	Time point of sampling *pi*[1]	Analyzed data[2]
1	Intestinal immune response	BALB/c	6	F	3 weeks	15 infected 5 negative controls	Week 1 Week 2 Week 3	Cyst excretion Intestinal cytokine levels
2	Intestinal immune response	C57Bl/6	6	F	3 weeks	15 infected 5 negative controls	Week 1 Week 2 Week 3	Cyst excretion Intestinal cytokine levels
3	Effect of IL17A on intestinal immune response	C57Bl/6 IL17RA-KO	6	F	3 weeks	5 infected IL17RA KO mice 5 infected controls	NA	Cyst excretion
4	Early intestinal immune response	C57Bl/6	6	F	1 week	35 infected 14 negative controls	Daily, starting day 1 *pi* until day 7	Intestinal cytokine levels

[1] samples were taken from the small intestine for subsequent qRT-PCR analysis of the main cytokines. [2] cyst counts in faeces were performed daily for each animal

3.2.4. Tissue sample collection.

At necropsy, 1 cm long fragments of the small intestine were taken from each animal starting 4 cm from the gastro-duodenal junction. To allow RNA extraction, the intestinal tissue was snap frozen in liquid nitrogen and stored at -80°C.

3.2.5. RNA isolation and quantitative real time PCR.

Total RNA was isolated by adding TRIzol (Invitrogen) to tissue followed by an RNA purification with the RNeasy mini kit (Qiagen). Any contaminating genomic DNA was removed with the help of the RNase-free DNase set (Qiagen) for on-column DNase digestion. An Experion™ Automated Electrophoresis System (Bio-Rad) was used to verify the quality of the isolated RNA, while total RNA

concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). To obtain the cDNA samples needed for quantitative Real-time PCR (qRT-PCR) the iScript cDNA synthesis kit was used following the manufacturer's instructions.

All qRT-PCR analyses were carried out using the SYBR Green Master Mix (Applied Biosystems), with 2 µl of single stranded cDNA (10 ng of the input total RNA equivalent) and 500 nM of amplification primer in a reaction volume of 20µl. The primer sets for amplification of the different genes were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/) and are listed in the appendix.

All analyses were run on a StepOnePlus Real-Time PCR system (Applied Biosciences) with the following amplification cycles: 95°C for 20 s; 35 cycles of 95°C for 5 s and 60°C (optimal annealing temperature for all the examined genes) for 30 s. Reaction efficiencies were measured based on a standard dilution curve obtained by serial dilutions of pooled cDNA material from all the samples. In every assay, a non-template control was added and cDNA samples were analyzed in duplicate. At the end of the reactions melting curve analyses were performed to ensure specificity of the primers. The delta Ct method was applied to transform the obtained Ct values in relative quantities. With this method, the following formula was used: $Q=E^{(\min Ct-sampleCt)}$ where Q represents sample quantity relative to the sample with highest transcription; E represents amplification efficiency; min Ct is equal to the lowest Ct value; and sampleCt is the Ct value of sample with the highest transcription level. The obtained quantities were then normalized against internal control genes, referred to as housekeeping genes (HKGs). Out of a panel of 6 candidate genes (beta actin (*Actb)*, hypoxanthine phosphoribosyltransferase 1 (*Hprt1*), ribosomal protein P0 (*Rplpo*), succinate dehydrogenase flavoprotein subunit A (*Sdha*), TATA box binding protein (TBP)-associated factor (*Taf2*), and ubiquitin-conjugating enzyme E2D2 (*Ube2d2*)) the most stable HKGs were selected based on the gene stability measure M calculated by the GeNorm software (geNorm3.5) (Andersen et al., 2004). The eventual gene expression normalization factor for each animal sample was calculated based on the geometric mean of the two selected HKG's. For the BALB/c animals, *Hprt1* and *Rplp*o appeared to be the most suitable HKG. In C57Bl/6 animals, this resulted in the use *of Hprt1* and *Tbp*. Gene expression was evaluated based on fold differences in gene transcription levels compared to the negative control animals. This was done by

calculating the ratios of individual relative quantities on the geometric means of relative values of the control samples.

Statistical analysis was carried out using GraphPad Prism software. A one way ANOVA followed by a Dunn's Multiple Comparison Test was used to determine differences between the infected and the control group. A P-value $\leq$ 0,05 was considered significant.

3.2.6. Histology

To determine the presence of trophozoites in the small intestine the tissue samples were stained with haematoxylin-eosine (HE).

3.3. Results

3.3.1. Analysis of the intestinal immune response in infected mice reveals a strong IL-17A up-regulation.

In a first trial, we analysed the intestinal immune response in *G. muris* infected BALB/c and C57Bl/6 mice. The daily faecal cyst excretion of the individual animals revealed a similar infection pattern as seen earlier in *G. muris* trials (Belosevic et al., 1984), with a peak number of cysts around day 5 *pi* for BALB/c and day 6 *pi* for C57Bl/6 animals (Figure 3.1). Afterwards, cyst shedding rapidly declined.

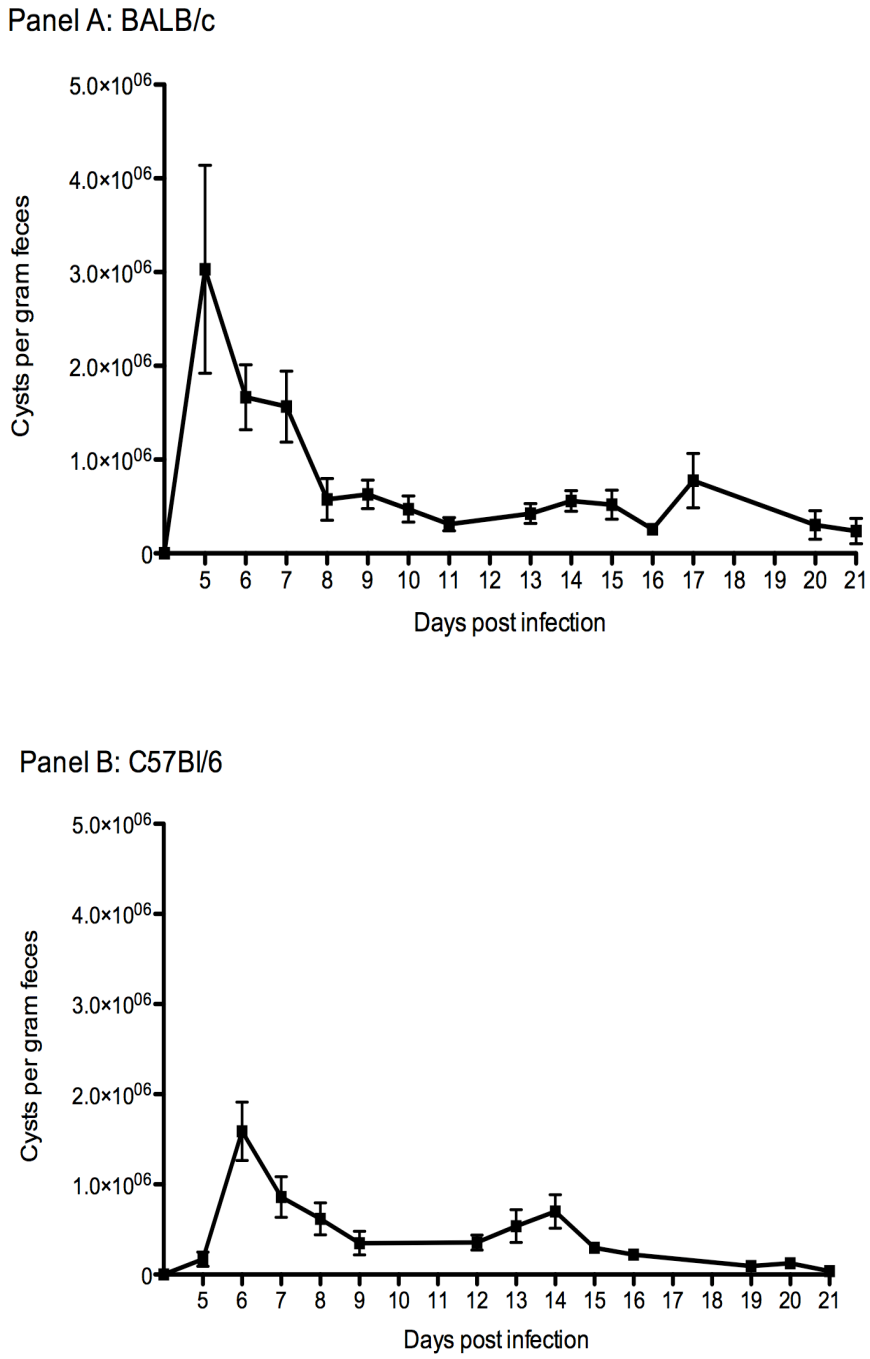

Figure 3.1: cyst excretion in *G. muris* infected BALB/c mice and C57Bl/6 mice
Cyst excretion was counted daily using a sucrose density technique. Each point on the graph represents the mean cyst output per gram feces of 5 animals ± standard error.

Next, we compared the gene transcription of a panel of cytokines in infected animals to non-infected controls. This panel consisted of the same cytokines looked at in

cattle, i.e. IFN-γ, IL-1β, Il-4, IL-6, IL-10, IL-13, IL-17A, RORγT, TGF-β1 and TNF-α. A qRT-PCR analysis revealed a striking transcriptional up-regulation of *Il17a* in both infected mice strains (Figure 3.2,). In BALB/c mice, the observed up-regulation started from week 1 *pi*, and further increased on weeks 2 and 3. Except for *Il10* at weeks 1 and 3 *pi*, no other cytokine showed a significantly up-regulated gene transcription in infected BALB/c mice compared to negative controls. A significant down-regulated transcription could be seen for *Ifng* at week 2 *pi* as well as a transcriptional down-regulation of *Tnfa.* In C57Bl/6 mice, the up-regulated expression of *Il17a* started at week 1 *pi* but only showed significance and a much higher fold change at week 3 *pi*. Except for *Il6* at week 1 *pi*, no other cytokine showed a significantly up-regulated gene expression in infected C57Bl/6 animals compared to negative controls (Figure 3.2, B). A significant down-regulated transcription could be seen for *Ifng* at week 3 *pi* in infected animals compared to controls. In addition, a down-regulation could be seen for *Tnfa* mRNA at week 1 *pi*.

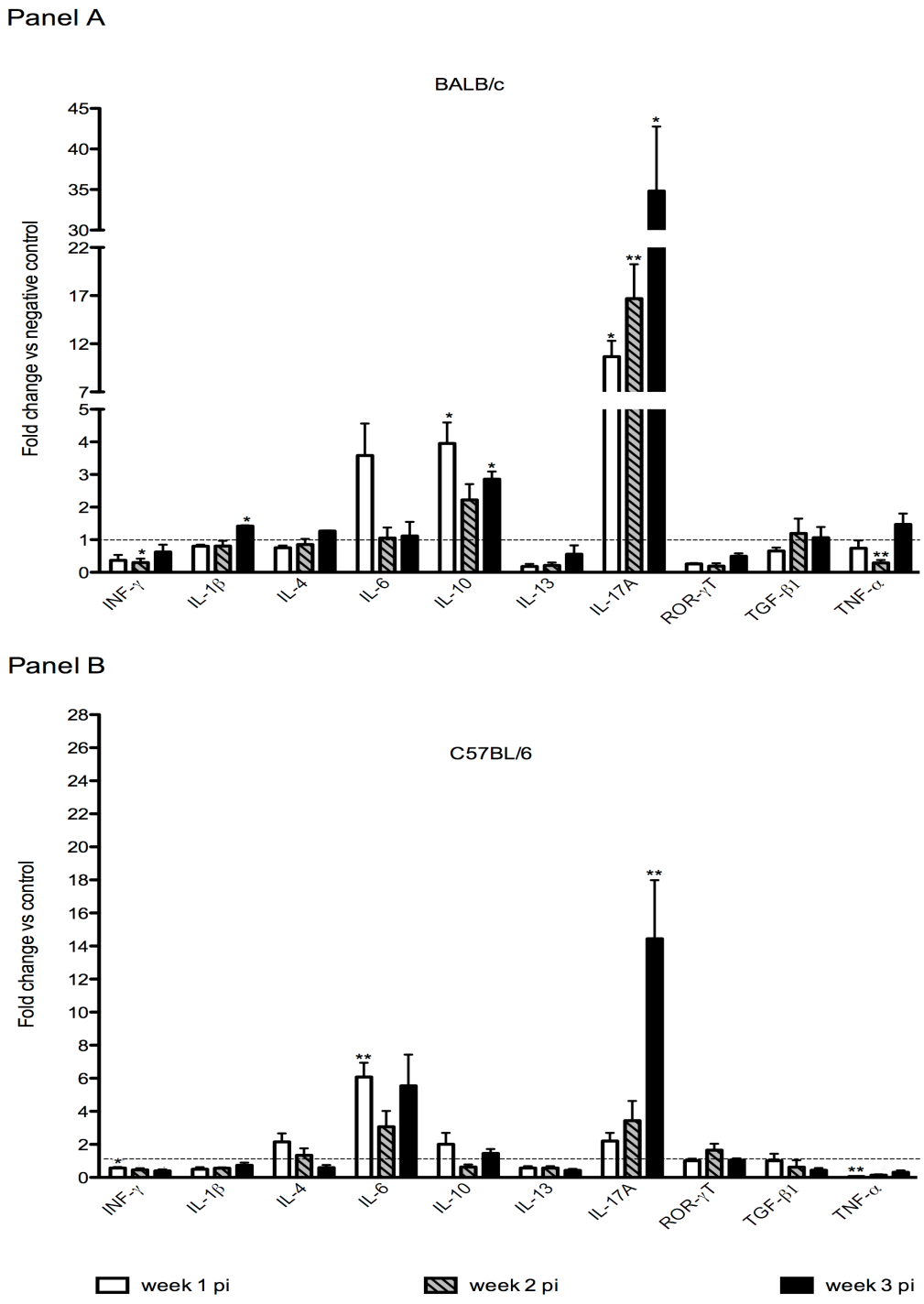

Figure 3.2. Cytokine transcription profile in the small intestine of *G. muris* infected BALB/c and C57Bl/6 mice.

Transcription levels of the main cytokines were analyzed using quantitative PCR. Mean fold changes (n = 5) in infected mice compared to uninfected controls are presented with SEM as error bars. (**P<0.01, *P<0.05)

3.3.2. Il-17 is required for clearance of G. muris.

Production of IL-17A in the intestine has been described before as critical in the host defence against a number of infections such as extra-cellular bacteria (reviewed in Korn et al., 2009). To demonstrate whether the transcriptional up-regulation of IL-17A is important in the immunity against *G. muris*, a new infection experiment was set up in mice deficient for IL-17 receptor A (IL-17RA) on a C57Bl/6J background (Ye et al., 2001). After infection of the IL-17R KO animals and C57Bl/6 wild-type controls, cumulative cyst counts were significantly higher in KO animals compared to controls (Figure 3.3, A). This effect persisted throughout the whole three-week duration of the study, after which WT animals were able to largely clear their infections while in KO animals high numbers of cysts persisted. In addition, to determine the presence of *G. muris* trophozoites in the gut lumen, samples were taken from the small intestine of all animals at day 21 *pi* for further histological examination. A haematoxylin-eosine staining (Figure 3.3, B&C) revealed that only a few trophozoites could be found after thorough microscopic examination of the whole slide in all WT animals, while a thick layer of trophozoites was still visible in the lumen of all the IL-17RA KO mice, indicating that the observed cyst counts are a result of the high number of trophozoites remaining in the gut lumen under circumstances where IL-17A is unable to execute its function.

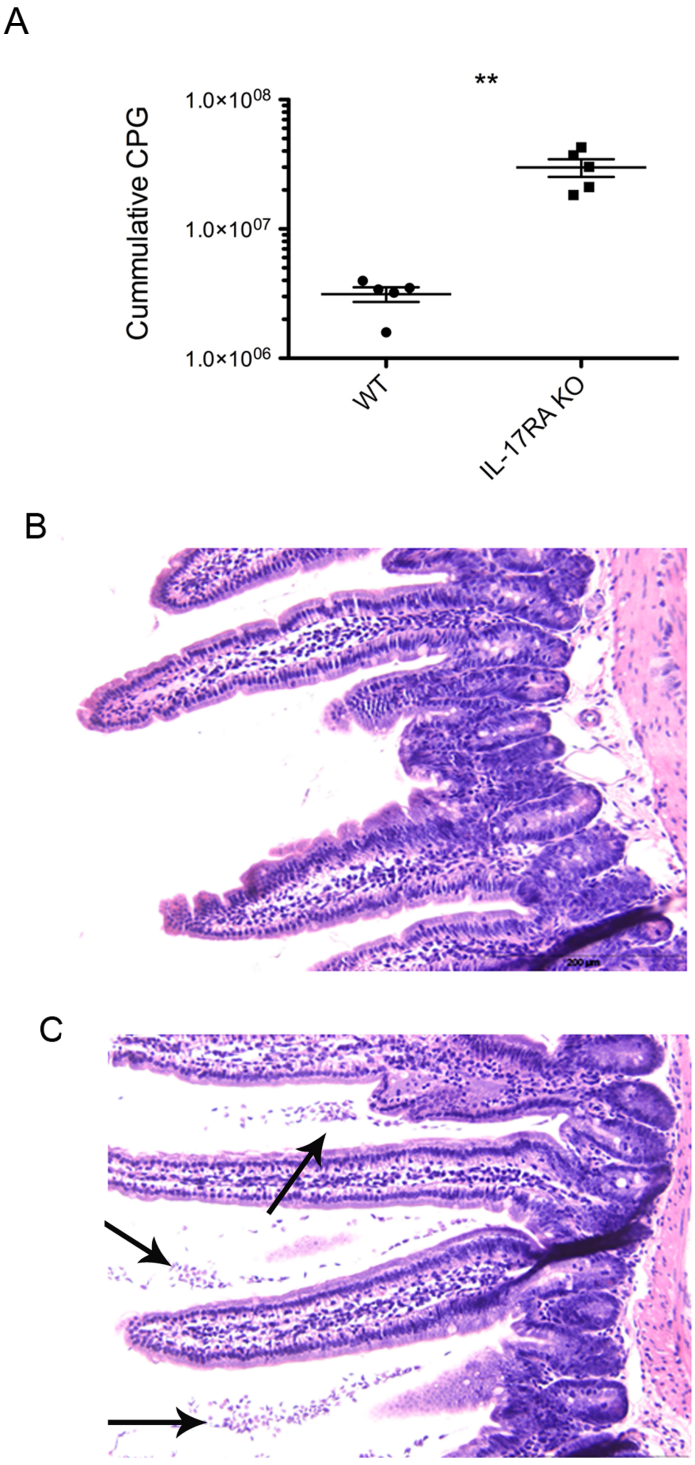

Figure 3.3. Cyst secretion and presence of trophozoites in *G. muris* infected IL17RA KO animals and wild-type controls. To analyze the effect of the observed *il17a* up-regulation, an infection trial was set up comparing IL-17RA KO animals to infected wild-type controls. Panel A shows the mean cummulative cyst output per gram feces of 5 animals ± standard error. ** = P<0,01. Panel B & C show a HE staining to illustrate the lack of trophozoites in the intestinal lumen of wild-type controls at week 3 *pi* (B), whith high numbers of trophozoites (arrows) still visible in the intestinal lumen of the KO animals (C).

It is noted in literature that the production of IL-17 in mice usually occurs in the presence of IL-6 and TGFβ. However, these cytokines were not found to be significantly up-regulated at week 1, 2 or 3 *pi* in both mouse strains, with the exception of *Il-6* at week 1 in C57Bl/6 animals. We hypothesized that the cytokines upstream of IL-17 may appear earlier on in the infection. We decided to investigate this more closely and to set up a new infection trial in which the intestinal response was analysed daily starting day 1 *pi* in C57Bl/6 animals. Both *Il-6* and *Tgfb1* were found to be significantly up-regulated preceding or coinciding the *Il-17a* up-regulation that started at day 6 *pi* (Figure 3.4). In line with its regulatory role, the transcription factor *Rorc* showed a higher transcription in the infected animals compared to the controls at the earlier time points.

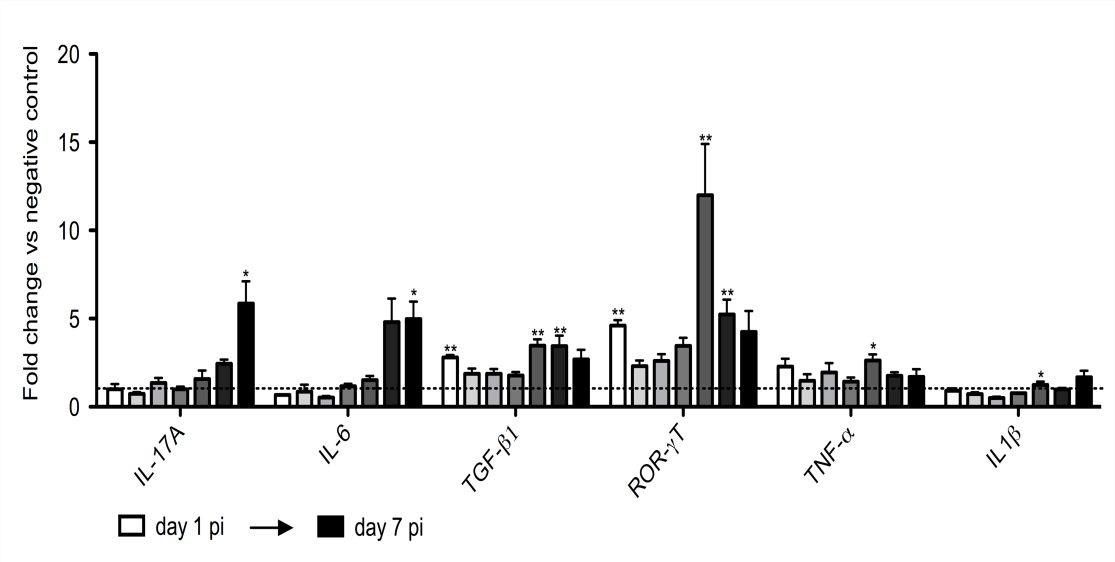

Figure 3.4. Transcription profile of cytokines in the small intestine of *G. muris* infected C57Bl/6 mice during the first days post infection. The transcription profile of cytokines was analyzed starting day 1 *pi* up to day 7. Mean fold changes (n = 5) in infected mice compared to uninfected controls are presented with SEM as error bars. (**P<0.01, *P<0.05)

As a first step towards unravelling the effector mechanisms downstream of IL-17A, qRT-PCRs were performed to examine the transcription of 6 antimicrobial peptides and proteins: the β-defensins (DEFB) 2 and 3, Reg3γ, calprotectin S100A8 and S100A9 and cathelicidin. An up-regulated transcription, although not significant, could be seen for both calprotectin *s100a8* and *s100a9* at week 1 *pi* (Figure 3.5). From the remaining genes analysed, cathelicidin was not differently expressed compared to control animals and no transcripts of the β-defensins and Reg3γ could be amplified or detected in any of the samples. This was however a small preliminary study and more thorough investigation of the intestinal transcriptome of both infected and non-infected IL-17RA KO and WT mice could provide more detailed information on the intestinal effector mechanisms induced by IL-17A production.

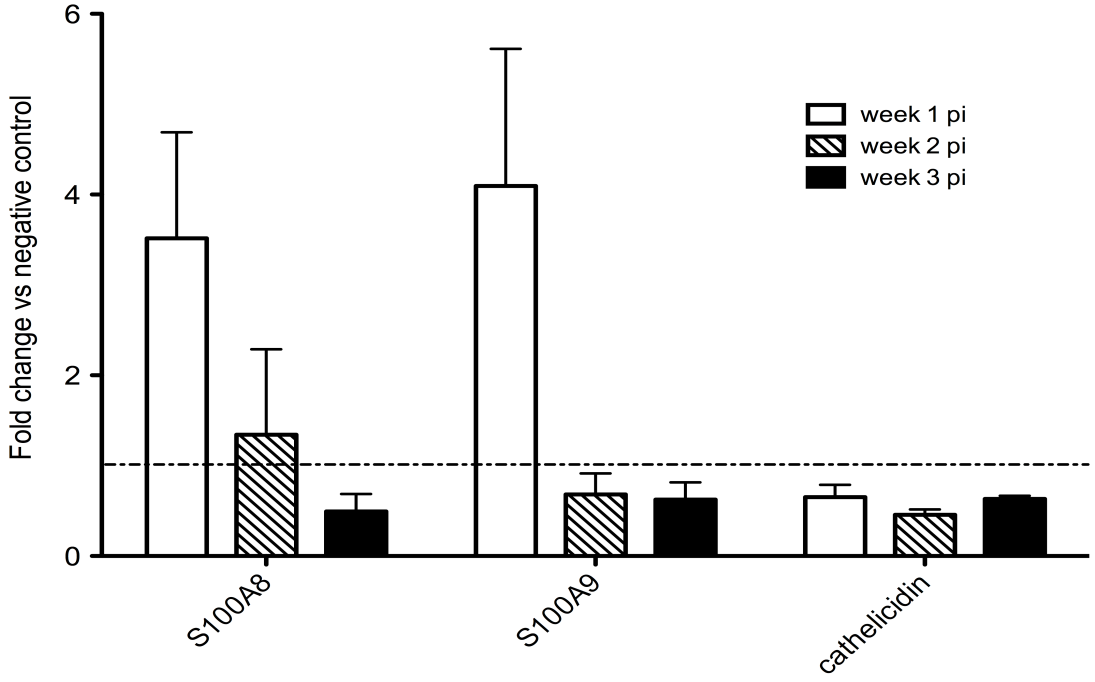

Figure 3.5. Transcription profile of antimicrobial proteins in small intestine of *G. muris* infected C57Bl/6 mice. Transcription levels of antimicrobial proteins were analyzed using quantitative PCR. Mean fold changes (n = 5) in infected mice compared to uninfected controls are presented with SEM as error bars. (**P<0.01, *P<0.05)

3.4. Discussion

In this study the general intestinal immune response in mice following a *G. muris* infection was investigated. The most prominent finding was the induction of IL-17A following infection, as measured on transcript level by qRT-PCR. The highest transcriptional up-regulation was repeatedly observed at week 3 post infection. Although some variation was apparent between the different experiments, the data indicated that IL-17A induction occurred from approximately 1 week p.i. onwards. In addition, when the functioning of this cytokine was impaired by deletion of IL-17RA in C57Bl/6 mice, infected animals could no longer dispose of the parasite. In the absence of littermate IL-17RA +/+ control mice, infection levels in the IL-17RA KO mice were compared to C57Bl/6 wild-type mice. Although mice from different litters potentially have a different gut microbiome, it is unlikely that this is causing the lack of immune development in the IL-17RA KO mice. Analysis of cyst secretions in C57BL/6 WT mice from different origins and in the BALBc mice all showed a similar pattern, with the infection almost disappearing at 3 weeks p.i., which is completely different from the pattern observed in the IL-17RA KO mice.

To our knowledge, this is the first time that IL-17A production itself is seen in the intestine of *G. muris* infected mice. Earlier work using the *G. muris* model did identify $CD4^+$ T cells as important elements in the clearance of the parasite, shown by the development of prolonged infections in nude mice (Stevens et al., 1978) and mice depleted of T helper cells (Heyworth et al., 1987). Since these cells were not further characterised, the possibility remains that these were $CD4^+$ Th17 cells. Interestingly, when looking at *G. duodenalis*, an earlier study described an elevated production of IL-17 in murine spleen and mesenteric lymph node cells in response to *G. duodenalis* extract (Solaymani-Mohammadi and Singer, 2011), suggesting that an IL-17 response could be a typical anti-*Giardia* response in mice. In addition, cattle infected with *G. duodenalis* showed a systemic IL-17 production after approximately 7 to 9 weeks of infection (Grit et al., 2014), at a time that protective immunity develops. Importantly, it should be noted that the Il-17RA not only functions as a receptor for IL-17A, but is also important for IL-17E and IL-17F signalling. The latter two cytokines however never showed an up-regulated transcription in infected mice compared to the controls.

Interleukin 17 is a cytokine that can be produced by cells that are both part of the innate as well as the adaptive immune system. In the intestine of mice, the lamina

propria already contains high numbers of $CD4^+$ Th17 cells that are present at steady state. Other possible less conventional IL-17 cellular sources include gut $ROR\gamma T^+$ γδ T cells, $CD8^+$ αβ T cells, invariant natural killer cells, lymphoid tissue inducer (LTi), LTi^- like cells and paneth cells (reviewed in Morrison et al., 2011).

The role of IL-17 in the intestine has been described as dual as it can work in both a protective and pathological manner. The way it serves as an effector cytokine during the clearance of a *G. muris* infection still needs to be further examined. A possible explanation is the stimulation of mucin and defensin production by IL-17 (Chen et al., 2003; Ishigame et al., 2009), both of which were earlier described as candidate defence mechanisms during *G. duodenalis* infection (reviewed in (Muller and von Allmen, 2005)). In our study in C57Bl/6 mice however, no significant changes could be observed in the expression levels of 6 antimicrobial peptides and proteins that are under the control of IL-17A (i.e. β defensins, Reg3γ, cathelicidin and calprotectin), suggesting that other factors are likely in play.

In summary, this chapter revealed that a Th17 like response with IL-17A production can be observed during a *G. muris* infection in both BALB/c and C57Bl/6 mice. The production of IL-17A appeared to be an important factor in the protective immune response as was demonstrated in the infection trial using IL-17 receptor A KO mice. Whereas in wild type mice cyst secretion dropped significantly after 3 weeks of infection, the IL-17RA-KO mice were unable to clear the infection.

3.5. References

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Chapter 4

Giardia muris infection and the Peroxisome Proliferator-Activated Receptors.

Based on: Dreesen L., De Bosscher K., Grit G., Staels B, Lubberts E, Bauge E., Geldhof P. (2014). *Giardia muris* infection in mice is associated with a protective IL-17A response and induction of the Peroxisome Proliferator-Activated Receptor Alpha. Infection and Immunity. IAI.01536-14.

4.1. Introduction

In chapter 2 we described the transcriptional up-regulation of the nuclear peroxisome proliferator-activated receptors (PPAR) alpha and gamma in the intestine of infected calves. Considering the anti-inflammatory potential of these receptors after activation (Daynes and Jones, 2002), this could possibly explain the chronicity of a *Giardia* infection in cattle. In *G. muris* infected mice (chapter 3), a clear IL-17 response was observed, in contrast to the downregulated IL-17A transcription in the calves. In addition, mice could easily clear the parasite after about 3 weeks of infection.

Following the hypothesis originating from the cattle work that the PPARs have a role in the chronicity of the infection and with the clear protective response in mice in mind, we would not expect an up-regulated expression of the PPARs in *G. muris* infected mice. In this chapter, the potential induction and activation of PPARs in the intestine of infected BALB/c and C57Bl/6 mice were investigated in order to further compare these responses in the different mice strains and to the cattle work.

4.2. Material and methods

4.2.1. Ethical statement.

All animal experiments were conducted in accordance with the E.U. Animal Welfare Directives and VICH Guidelines for Good Clinical Practice, and ethical approval to conduct the studies were obtained from the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, who have also approved the document.

4.2.2. Mouse studies.

All *in vivo* experiments performed are summarized in table 4.1. The BALB/c and C57Bl/6 animals described in chapter 3, experiment 1 and 2 were used again in this chapter. In addition, the samples obtained from the animals used in the early immune response work in chapter 3, experiment 4, were used again in this chapter.

An additional infection experiment (experiment 5) compared *Ppara* and *Pparg* expression during 7 days of infection in infected BALB/c mice to non-infected

BALB/c controls.

Again in BALB/c animals, experiment 6 used a total of 5 BALB/c mice (Harlan laboratories) that received the PPARα agonist fenofibrate (Sigma Aldrich). This was given to them by daily oral gavage suspended in olive oil (200mg/ml), at a dose of 800mg/kg body weight. An additional 5 animals were infected with *G. muris* in combination with the daily agonist administration. A third group was infected without additional agonist intake and a final group received no agonist or parasite. The latter two groups both received the olive oil vehicle only. Cyst excretion was counted daily and intestinal samples were taken after 3 weeks of infection.

In a final experiment (number 7) the course of a *G. muris* infection in female PPARα KO animals (Lee et al., 1995) (of a 10 generations backcrossed C57BL/6N genetic background) and in C57Bl/6 WT controls was compared during 3 weeks of infection. To achieve this, 5 KO animals and 5 WT mice were infected with *G. muris* cysts after which cyst counts were performed daily for each animal.

4.2.3. Cyst counts and tissue sample collection.

To monitor the course of a *G. muris* infection in all trials, cyst excretion in faeces was measured as described previously by Roberts-Thomson et al. (1978) (Roberts-Thomson and Mitchell, 1978) and in chapter 3. Cyst excretion was then expressed as number of cysts per gram faeces. In the PPARA KO and agonist trials, statistical analysis was done using GraphPad Prism software. All cyst counts from one animal were considered as repeated measures and a two way repeated measures ANOVA with Bonferroni posttest was used to calculate differences at every time point between the groups.

At necropsy, 1 cm long fragments of the small intestine were taken from each animal starting 4 cm from the gastro-duodenal junction. To allow RNA extraction, the intestinal tissue was snap frozen in liquid nitrogen and stored at -80°C.

Table 4.1. summary of mice experiments described in chapter 4

Expt nb	Objective	Strain	Age (w)	Sex	Trial duration	Number of animals	Time point of sampling *pi*[1]	Analyzed data[2]
1	Intestinal immune response	BALB/c	6	F	3 weeks	15 infected 5 negative controls	Week 1 Week 2 Week 3	Cyst excretion Intestinal PPAR levels
2	Intestinal immune response	C57Bl/6	6	F	3 weeks	15 infected 5 negative controls	Week 1 Week 2 Week 3	Cyst excretion Intestinal PPAR levels
4	Early intestinal immune response	C57Bl/6	6	F	1 week	35 infected 14 negative controls	Daily, starting day 1 *pi* until day 7	Intestinal PPAR levels
5	Early intestinal immune response	BALB/c	6	F	1 week	35 infected 14 negative controls	Daily, starting day 1 *pi* until day 7	Intestinal PPAR levels
6	Effect of PPARα agonist on intestinal immune response	BALB/c	6	F	3 weeks	Infected: 5 plus agonist, 5 without Negative controls: 5 plus agonist, 5 without	Week 3	Cyst excretion Intestinal cytokine levels
7	Effect of PPARα deletion on the intestinal immune response	C57Bl/6 PPARα KO	6	F	3 weeks	5 infected PPARα KO mice 5 infected controls	NA	Cyst excretion

[1] samples were taken from the small intestine for subsequent qRT-PCR analysis of the main cytokines. [2] cyst counts in faeces were performed daily for each animal

4.2.5. RNA isolation and quantitative real time PCR.

Total RNA was isolated by adding TRIzol (Invitrogen) to the snap-frozen tissue followed by an RNA purification with the RNeasy mini kit (Qiagen). Any contaminating genomic DNA was removed with the help of the RNase-free DNase set (Qiagen) for on-column DNase digestion. An Experion^{TM} Automated Electrophoresis System (Bio-Rad) was used to verify the quality of the isolated RNA, while total RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). To obtain the cDNA samples needed for quantitative Real-time PCR (qRT-PCR) the iScript cDNA synthesis kit was used following the manufacturer's instructions.

All qRT-PCR analyses were carried out as described in chapter 3. Statistical analysis was carried out using GraphPad Prism software. A one way ANOVA followed by a Dunn's Multiple Comparison Test was used to determine differences between the infected and the control group. A P-value ≤ 0.05 was considered significant.

4.3. Results

4.3.1. Peroxisome proliferator-activated receptor (PPAR) α gene transcription is up-regulated early on in infection in C57Bl/6 mice

A qRT-PCR analysis was used to check the transcription profile of both *Pparg* and *Ppara* (Figure 4.1.) in BALB/c and C57Bl/6 mice. In BALB/c animals, no up-regulation of *Pparg* could be seen, and transcription was even significantly down-regulated at week 2 *pi*. Concerning *Ppara* and its downstream genes, no significantly increased transcription was observed in infected animals. In C57Bl/6 mice, like in BALBc's, no up-regulation could be seen whatsoever for *pparg* transcription. Concerning *Ppara*, C57Bl/6 mice showed up-regulations bordering the 2-fold threshold at week 1 *pi* and over 2-fold at week 2 and 3, although not statistically significant.

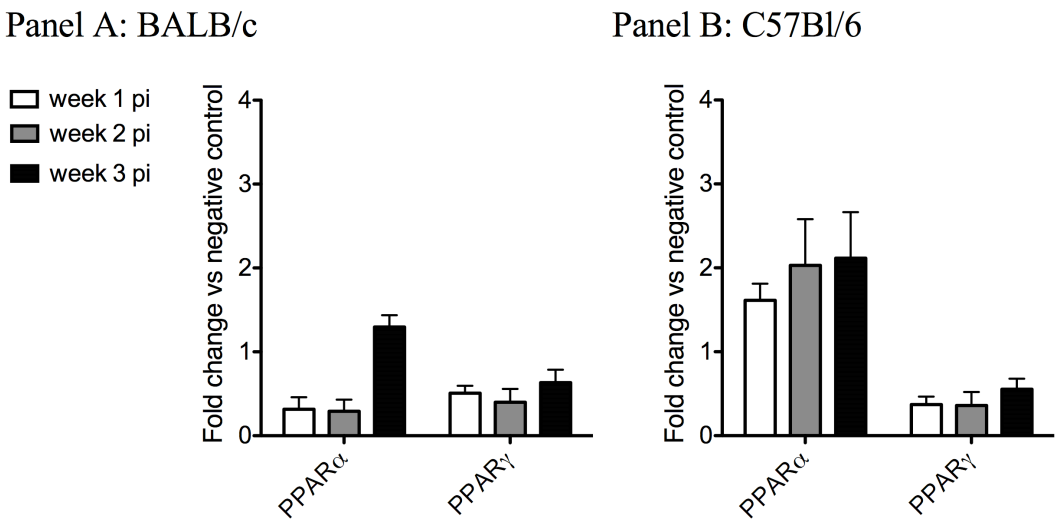

Figure 4.1.: PPAR transcription profile in small intestine of *G. muris* infected BALB/c and C57Bl/6 mice.

Transcription levels of the PPARs were analyzed using quantitative PCR. Mean fold changes (n = 5) in infected mice compared to uninfected controls are presented with SEM as error bars.

Additional qRT-PCR analyses of *Ppara* were done on the intestinal samples collected daily from C57bl/6 animals starting day 1 *pi* up until day 7 *pi* (mentioned in chapter 3). This indicated a transcriptional up-regulation of *Ppara* from day 1 *pi* onwards in the infected animals compared to the negative controls (Figure 4.2). To investigate whether a possible up-regulation of *Ppara* expression also results in a functional PPARα activation, three genes (pyruvate dehydrogenase kinase, isozyme 4 (*Pdk4*), carnitine palmitoyltransferase 1a (*Cpt1*) and phospholipid transfer protein (*Pltp*)) that are known downstream targets of PPARα (Lefebvre et al., 2006) (typically measured in liver samples) were included in the qRT-PCR analysis. An up-regulation was observed for 2 of the downstream target genes, with a significant up-regulation of *Pdk4* and *Pltp* mRNA levels starting day 1 *pi*, suggesting that the observed *Ppara* up-regulation could indeed be linked to a functional PPARα activation. No up-regulation of *Pparg* expression could be observed. Repeating the same experiment in BALB/c animals revealed no significant up-regulation of *Ppara, Pparg* or the downstream target genes at any timepoint (Figure 4.3.)

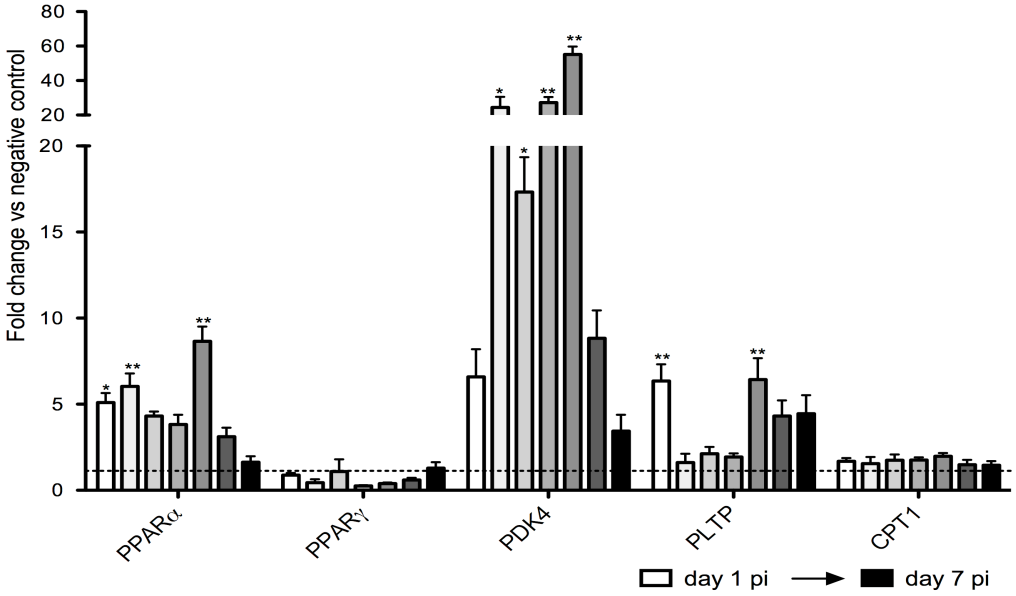

Figure 4.2. Transcription profile of PPARα and the main downstream target genes in the small intestine of *G. muris* infected C57Bl/6 during the first days post infection. The transcription profile of of *Pparγ*, *Pparα* and the selected downstream target genes was analyzed starting day 1 *pi* up to day 7. Mean fold changes (n = 5) in infected mice compared to uninfected controls are presented with SEM as error bars. (**P<0.01, *P<0.05)

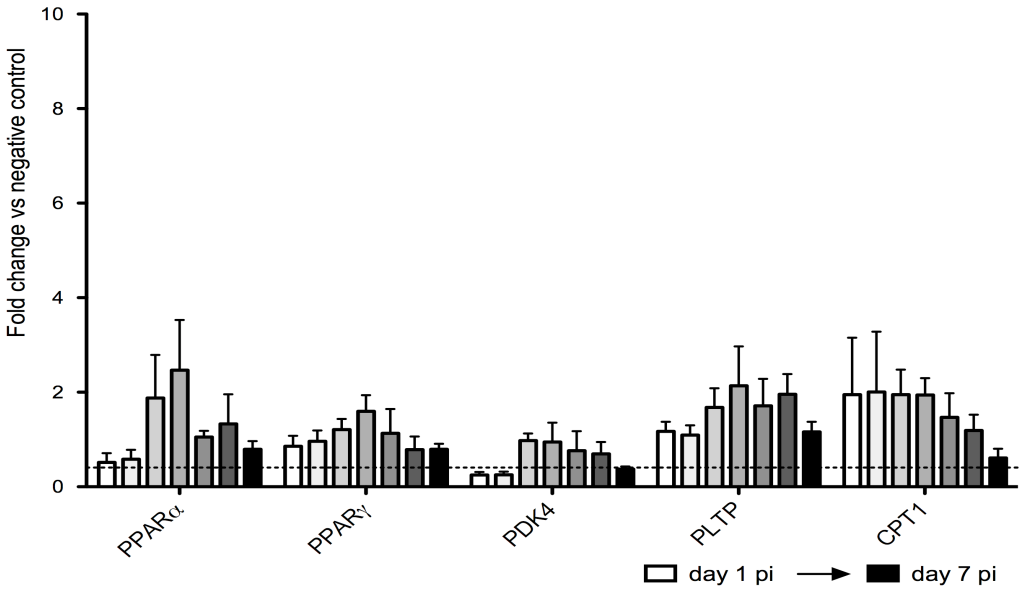

Figure 4.3. Transcription profile of PPARα and the main downstream target genes in the small intestine of *G. muris* infected BALB/c during the first days post infection. The transcription profile of *Pparγ*, *Pparα* and the selected downstream target genes was analyzed starting day 1 *pi* up to day 7. Mean fold changes (n = 5) in infected mice compared to uninfected controls are presented with SEM as error bars.

4.3.2. PPARα might influence cyst excretion numbers, but has no influence on infection duration or immune development.

To unravel any potential influence of PPARα on a *Giardia muris* infection, two approaches were used. Since no PPARα activation was observed in BALB/c mice, a PPARα agonist was used to induce activation of the receptor. Since C57Bl/6 animals did show an upregulated transcription of PPARα, an infection experiment was set up where C57Bl/6 animals with a deletion of PPARα were infected.

The BALB/c mice were given a PPARα specific agonist (fenofibrate) in combination with *G. muris*. Infected mice were given their daily dose of fenofibrate, whilst another group received the parasite but not the agonist. In addition, one group of animals received neither the agonist nor the parasite and served as a negative control group. To check if the agonist indeed resulted in an upregulated transcription of *Ppara* in the intestine, an agonist control group consisting of non-infected animals receiving only the agonist was introduced. As shown in figure 4.4, administration of the agonist led to noticeable lower cyst counts than those observed in the infected control animals. Both groups were nonetheless able to clear the infection by day 21, indicating that PPARα had no influence on the final outcome of a protective immune response and thus the (timing of the) eventual elimination of *G. muris*.

Comparison of the qRT-PCR data (Figure 4.5, panel A) obtained from the fenofibrate treated infected group with the negative control showed a significant up-regulation of *Ppara* transcription after 3 weeks of infection. In contrast to the first infection trial, the elevated *Ppara* transcription could also be seen in the infected BALB/c animals that did not receive any agonist. Looking at the downstream genes *Pdk4*, *Pltp* and *Cpt1*, all showed a higher transcription, with a significant result for *Cpt1* in the infected agonist group.

The calculated fold changes for cytokine transcription in the different groups suggest that PPARα agonist administration might have no or little effect on cytokine production as *Il17a* transcription was still up-regulated in the infected groups compared to the controls, although with markedly lower fold changes than those seen in earlier trials.

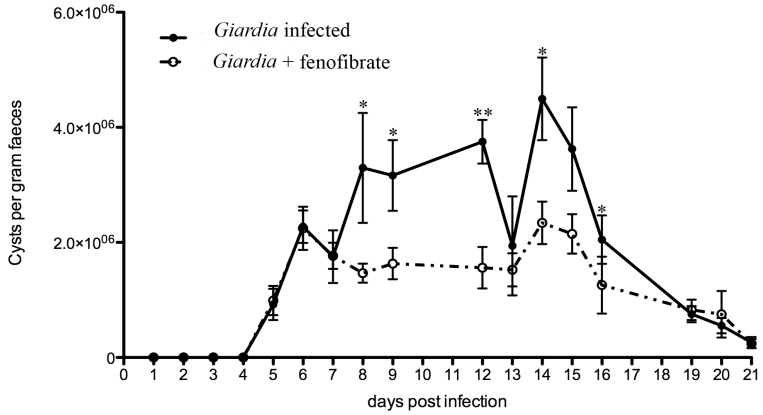

Figure 4.4. Cyst excretion in fenofibrate treated BALB/c animals.

Cyst counts were performed daily starting day 5 *pi* until 3 weeks *pi*. Each point on the graph represents the mean cyst output per gram feces of 5 animals ± standard error.

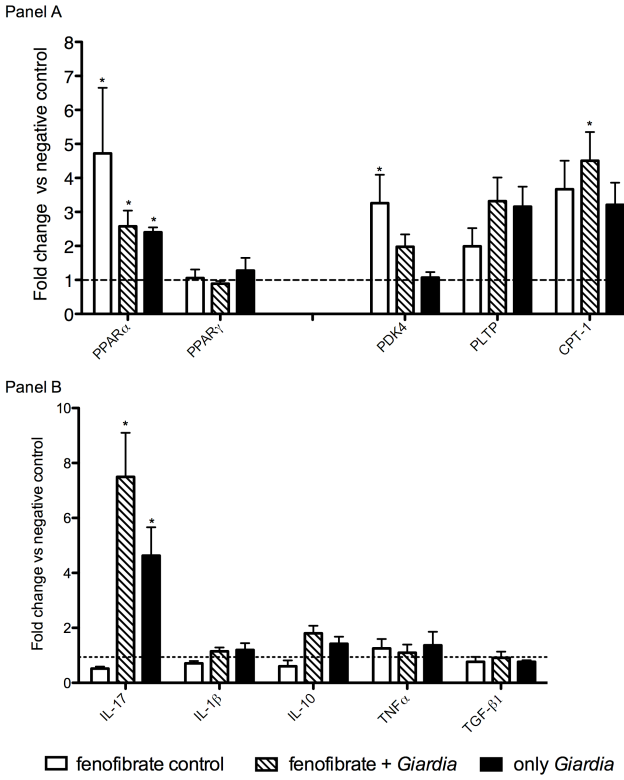

Figure 4.5. Intestinal transcription profile in fenofibrate treated BALB/c animals. Quantitative RT-PCR was used to analyze the transcription profile in the intestine of *G. muris* infected animals administered with the PPARα agonist fenofibrate (agonist + *Giardia*), infected mice without agonist administration (only *Giardia*) and uninfected mice treated with the agonist (agonist control). Mean fold changes (n = 5) at week 3 *pi* when comparing previous groups to uninfected untreated controls are presented with SEM as error bars (*P<0.05)

In a second experiment we compared the time course of infection between C57BL/6 WT and C57Bl/6 PPARα KO mice by counting the cyst excretion in both groups. To achieve this, cysts were counted daily starting day 5 pi up to week 3. Overall, no clear difference could be seen in the amount and duration of cyst excretion apart from day 5 *pi*, when cyst excretion in the KO animals was higher than what was seen in the control mice (Figure 4.6A) and the opposite effect on day 7 and 8 *pi,* although only statistically significant at day 8. To investigate whether cyst excretion already occurred earlier than day 5 pi in the PPARα KO mice, a second experiment was performed in which cyst excretion was measured daily starting from day 1 *pi* till day 7 *pi*. The results of this experiment are shown in figure 4.6B. Similar as in the first experiment, cyst excretion was detectable from day 5 *pi* onwards in the PPARα KO mice, whereas cyst excretion in the WT animals only started from day 6 *pi* onwards. On day 7 however, no more difference could be seen between the groups.

Quantitative RT-PCR analyses in both the WT and KO mice revealed that after deletion of PPARα, *Il17a* transcription was still significantly upregulated at week 1 and 3 *pi* in KO mice compared to uninfected negative controls. The WT animals that were included in this study again showed the up-regulated expression of Il-17A that was observed in the earlier trials at both week 1 and statistically significant at week 3 *pi* with a fold change of respectively 7 and 11 compared to negative WT controls (Figure 4.7).

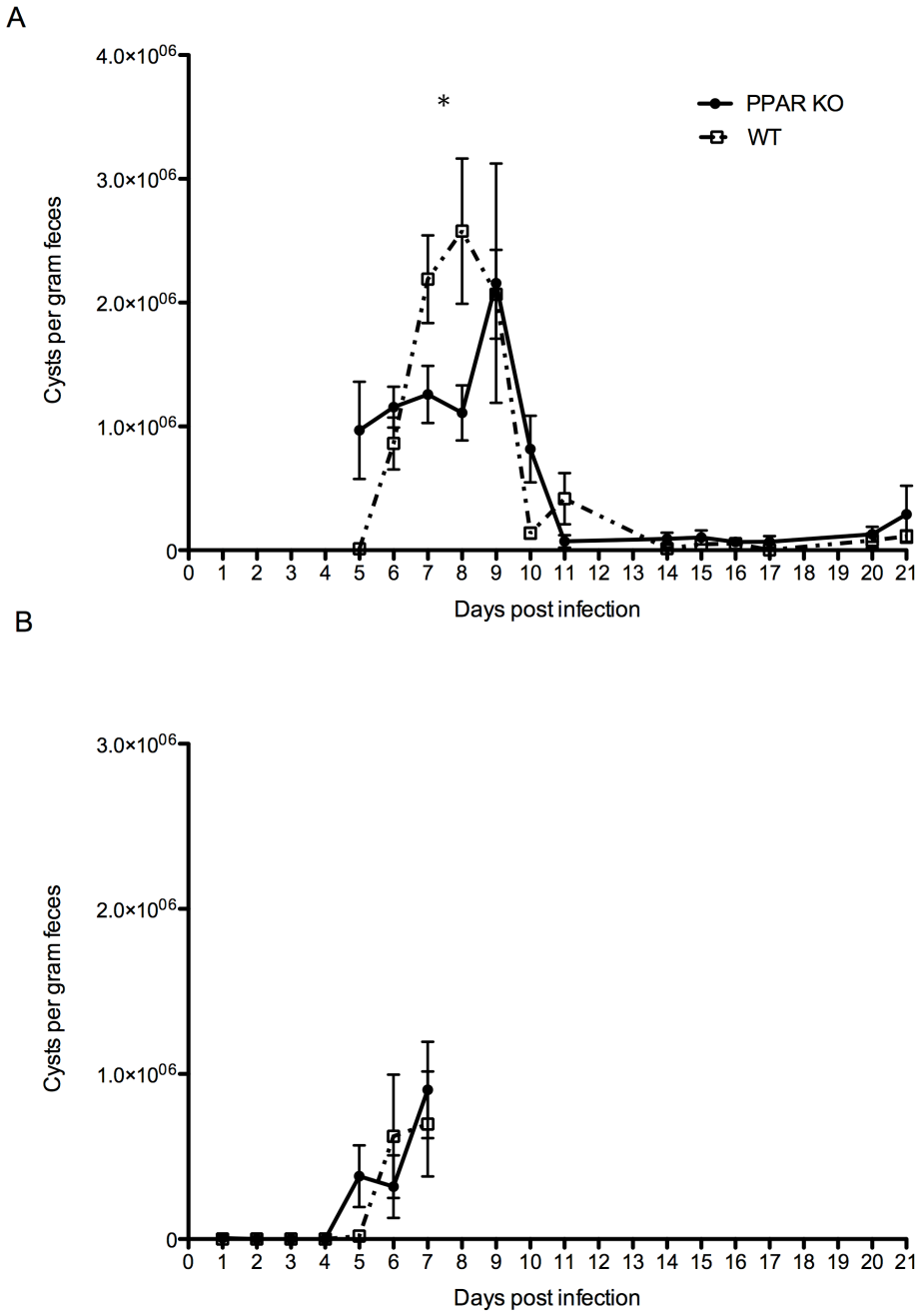

Figure 4.6. Comparison of infected PPARα KO mice and WT controls. Panel A shows the cyst counts of the first trial where counting started at day 5 *pi*, panel B shows the results of a second trial where counting was done daily starting day 1 *pi* untill day 7. Each point on the graph represents the mean cyst output per gram feces of 5 animals ± standard error.

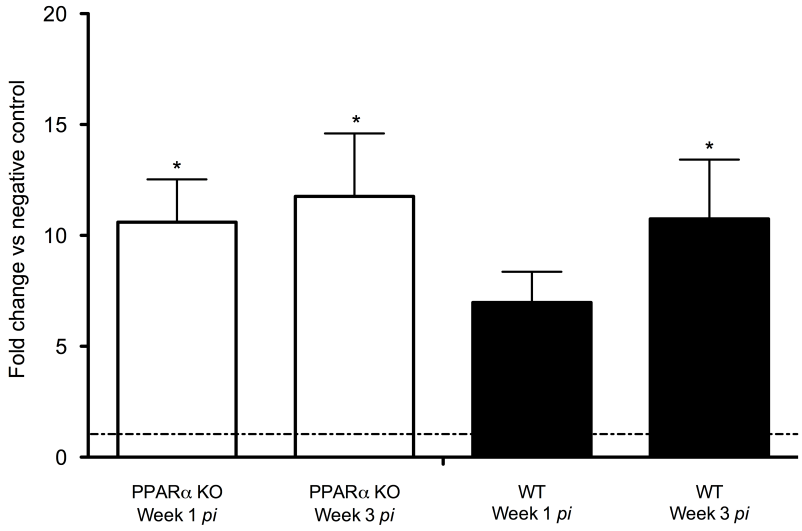

Figure 4.7. Transcription levels of *il17a* in PPARα KO and WT C57Bl/6 mice. Quantitative RT-PCR was used to analyze the transcription profile in the intestine of *G. muris* infected C57Bl/6 PPARα KO and WT mice. Mean fold changes (n = 5) at week 1 and 3 *pi* calculated by comparing previous groups to uninfected untreated controls are presented with SEM as error bars (*P<0.05).

4.4. Discussion

In this chapter, the possible activation and role of PPARα and γ in *G. muris* infected mice was investigated. The qRT-PCR analysis on intestinal samples showed that both *ppara* and *pparg* transcription was not up-regulated in infected BALB/c mice. However, an up-regulated *Ppara* transcription could be seen after 3 weeks of infection in a different experiment. The qRT-PCR analysis in C57Bl/6 mice indicated that PPARα mRNA, but not PPARγ, was up-regulated in infected mice as soon as day 1 *pi*. The exact mechanism by which PPARα is induced and activated during an infection is still unclear since a variety of ligands produced by the host or the parasite could be responsible (discussed in chapter 2).

It appears that PPARα activation in BALB/c mice might have an influence on the number of cysts that are excreted by infected mice. This was shown by the lower cyst counts in animals receiving an agonist compared to non-agonist controls. In C57Bl/6 animals, a clear effect on cyst counts was not as apparent. However, in these animals, higher transcription levels of PPARα were observed than in BALBc's, while the established peak of cyst excretion remained lower and appeared one day later. In

addition, cyst secretion during the 3 weeks period of the study in the PPARα KO animals was not majorly affected by the absence of PPARα, although a higher cyst excretion was repeatedly observed on day 5 *pi* in the KO animals compared to WT mice. How PPARα could influence cyst production is still unclear but it is possible that the levels of cholesterol, bile acids and bile salts in the intestinal lumen have a role in this. All these factors have been shown to influence *Giardia* encystation (Adam, 2001) and are also a target or a part of the various metabolisms that are influenced by PPARα (Valasek et al., 2007).

As stated before, the PPARs can exert a suppressive effect on the immune response. We previously hypothesised that this effect might cause the chronic infection witnessed in calves infected with *G. duodenalis*, since in these animals a lack of immune respons was seen together with an up regulated transcription of PPARα and PPARγ. However, administration of the PPARα agonist to BALB/c animals did not exert any effect on the duration of an infection, since both the agonist-treated and the control animals were able to clear the infection after approximately 3 weeks. This was further confirmed in the PPARα KO experiment, since the KO animals were able to clear the infection similar as the WT mice.

Whether the PPARα activation has any influence on the kinetics of the IL-17A response early in the infection is still unclear, but it is interesting to note that the IL-17A production in C57Bl/6 animals only starts when the PPARα activation is largely gone. However, the deletion of PPARα does not seem to have a great effect on IL-17A expression considering that *il17a* still showed an up-regulated expression in PPARα KO animals, and agonist administration to BALB/c animals did not lower *Il17a* transcription. Further research is needed to unravel this potential regulatory role of PPARα in the IL-17 response.

It should be noted that the role of PPARγ likewise warrants further research. Several studies have previously shown the association between PPARγ and Th17 response suppression (Klotz et al., 2009; Park et al., 2009; Viladomiu et al., 2012) and earlier work in *G. duodenalis* infected cattle revealed an up-regulation of PPARG together with a significant down-regulation of IL-17A (chapter 2). Since no PPAR γ activation was observed in *G. muris* infected mice, it would be interesting to in future adopt a reverse strategy by activating PPAR γ in mice and subsequently monitor possible effects on *G. muris* and immune development. Unfortunately, initial

experiments with agonists failed to activate PPAR γ in the intestine of mice (results not shown).

In summary, this study revealed an early induction of PPARα in the intestines of *G. muris* infected C57Bl/6 mice. This upregulated expression was not seen at later time points in BALB/c mice, but needs to be examined at the earlier stages of infection. The upregulated transcription of *Ppara* could possibly influence the number of cysts that are excreted in the faeces of the host. It does not however seem to influence the duration of an infection or the intestinal host response. Further research is now needed to elucidate the cells and mechanisms involved in the induction and regulatory functions of PPARα.

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Chapter 5
General discussion

The objective of this thesis was to understand the origin of the chronicity observed in cattle infected with *Giardia duodenalis* by performing a detailed analysis of the intestinal response. In addition, the immune response in *G. muris* infected mice was looked at and compared with the findings in *G. duodenalis* infected cattle. In this final chapter, the results of these studies will be discussed as well as the future prospects of this research. In addition, an idea will be given of the possible applications of this thesis.

5.1. *Giardia* spp. and interleukin 17.

Infection with *G. muris* induced a strong up-regulation of IL-17A in the intestine of both BALB/c and C57Bl/6 mice. The importance of IL-17A in orchestrating the protective immune response was later demonstrated in an infection trial using IL-17 receptor A KO mice: whereas in wild type mice cyst secretion dropped significantly after 3 weeks of infection, the IL-17RA-KO mice were unable to clear the infection. In cattle infected with *G. duodenalis*, we observed a suppression of IL-17A during acute infection (chapter 2). However, additional work done in our lab by Grit *et al*. (2014) investigated the kinetics of the immune response in infected animals until they showed immune protection against a re-infection, which occurred after approximately 3 months of infection. The first signs of an adaptive immune response only became visible after approximately 9 weeks of infection, as characterized by a significant proliferation of IL-17A producing $CD4^+$ αβ T cells following *in vitro* re-stimulation of peripheral blood mononuclear cells (PBMC) with *G. duodenalis* antigens.

IL-17A is a rather unique cytokine that bridges innate and adaptive immunity. Its role in the intestine has been described as dual with both protective and pathological effects. The cytokine can be produced by the more conventional CD4+ αβ T cells or by a range of innate immune cells (such as amongst others LTi cells, γδ T cells and paneth cells) that can act as sentinels of the immune system and reside in the intestinal lamina propria and intraepithelial compartment close to the host-environment interface (Cua and Tato, 2010). Another possible IL-17 producing cell population in the gut are the paneth cells (Takahashi et al., 2008). In mice specifically, intestinal production of IL-17A could be attributed to γδ T cells, which can provide an early innate IL-17 response since toll like receptors can directly activate mouse γδ T

cells to induce IL-17 (Gibbons and Spencer, 2011). Similar to Th1 or Th2 cells, differentiation of $CD4^+$ αβ T cells into IL-17A producing cells requires T cell receptor recognition of its cognate antigen presented on major histocompatibility complex class II by professional antigen-presenting cells such as dendritic cells (DCs) combined with the production of IL-6 and TGF-β (Korn et al., 2009). Unlike Th17 cells, antigenic priming is not required for activation of the innate immune cells, for which IL-23 stimulation, produced by antigen presenting cells, is sufficient to switch on IL-17A secretion (Cua and Tato, 2010). The data obtained so far suggests that the observed IL-17A production following infection is a result of a Th17 response. In immune cattle, *in vitro* exposure of PBMCs to *Giardia* trophozoites resulted in the proliferation of IL-17A producing $CD4^+$ αβ T-cells (Grit et al., 2014). In mice, the intestinal up-regulation of IL-17A was preceded by increased expression of IL-6, TGF- β and ROR-γt, which are required to induce IL-17A expression in T-cells (Gibbons and Spencer, 2011). In addition, $CD4^+$ T cell deficient mice are unable to clear a *Giardia* infection (Singer and Nash, 2000). However, additional experimental evidence is needed to fully confirm this hypothesis.

Since our attempted immunohistological stainings for IL-17A on intestinal tissue of infected mice were not successful, further research is needed to identify the real cellular source. A possible approach is the use of RORγt GFP transgenic mice in a new infection trial. After infection, the number of GFP-positive cells can be quantified and phenotyped using flow cytometry. In addition, the IL-17A response following *Giardia* infection in cattle was only investigated and observed in acutely infected calves (chapter 2) and systematically in long term infected animals (Grit et al., 2014). Future work on these animals regarding IL-17A could therefore first focus on analysing the intestinal expression in chronically infected cattle. Afterwards, the cellular source of IL-17 production in the intestine can be identified using flow cytometry.

In general, IL-17A is critical for mucosal and epithelial host defense against extracellular pathogens by enhancing the production of tight junction proteins, mucins, antimicrobial proteins and chemokines (Cua and Tato, 2010). Previous studies have alluded to the importance of all these factors in the defence against *Giardia*, but *in vivo* evidence is still missing (Muller and von Allmen, 2005). On the other hand, it has also been reported that IL-17A can contribute to chronic inflammation associated with a parasitic infection. In the C57Bl/6 mice study, no

significant changes could be observed in the expression levels of a panel of antimicrobial peptides and proteins that are under the control of IL-17A (i.e. β defensins, Reg3γ, cathelicidin and calprotectin), suggesting that other factors are likely in play. This was however a small preliminary study and more thorough investigation of the intestinal transcriptome (using an RNA-seq approach) of both infected and non-infected IL-17RA KO and WT mice could provide more detailed information on the intestinal effector mechanisms induced by IL-17A production. The functional role of possible effector molecules brought forward by the RNA-seq analysis can then be further investigated by performing infection experiments in KO mice, pending availability. When the results still point to antimicrobial peptides, their effect on the viability of life trophozoites could be analysed in culture. The differential expression of the genes and transcripts that are thus identified in mice could also be analysed in the mucosal samples collected from the immune calves using qRT-PCR.

5.2. *Giardia* spp. and the peroxisome proliferator –activated receptor alpha

In the second chapter of this thesis, it became clear that an infection with *G. duodenalis* in cattle coincided with a concomitant intestinal PPARα and PPARγ up-regulation. These nuclear receptors are ligand activated transcription factors that comprise three isotypes (α, β/δ and γ) differing in tissue distribution and cellular functions (Michalik et al., 2006). All of these receptors combine an ability to modulate lipid and glucose homeostasis with the regulation of innate and adaptive immune responses (Wahli and Michalik, 2012). When the infection trials were repeated using *G. muris* in mice, an early up-regulation of PPARα could be seen in C57Bl/6 mice. In mice infected with *G. muris*, no up-regulated expression of *pparg* occurred. In addition, the *ppara* expression disappeared within one week of infection, followed by a Th17 response. In BALB/c mice, PPARα did not show an up-regulated expression at any timepoint during the initial infection experiment. It should however be noted that in the agonist trial, *G. muris* infected animals that were not administered with fenofibrate did show an up-regulated expression of PPARα at week 3 *pi*.

A number of endogenously present metabolites can induce and activate PPARα, such as the fatty acids and their derivatives (Daynes and Jones, 2002). It is unclear which are the ligands responsible for the observed PPARα activation during a

Giardia infection. The question remains if the PPARα activation observed in cattle and mice is secondary to the infection and triggered by host fatty acid metabolites or instead by lipids of the parasite. A possible approach to identify if lipids extracted from *Giardia* are acting as ligands for PPARα would be to combine an *in vitro* enterocyte culture system and a PPARα-dependent transcriptional system (Raspe et al., 1999).

One of the many biological effects that are attributed to the PPARs is their influence on inflammation and immunity. In both *in vitro* and *in vivo* models, the PPARs have shown a potent anti-inflammatory effect, shown for example in PPAR KO mice. The PPARs can influence the immune response through transrepression mechanisms that can suppress the activity of many transcription factors such as NF-κB, STATs and AP1 (Straus and Glass, 2007). There are different modes of action available to the PPARs to execute this transrepression, which depend more on protein-protein interaction than the more classical protein-DNA interactions that are attributed to transcription factors. First, the use and sequestration of certain co-activators by the PPARs makes them unavailable for other transcription factors and secondly PPAR-RXR heterodimers have the ability to bind to other transcription factors, resulting in cross-inhibition. Thirdly, PPARs can interfere with mitogen-activated protein kinase (MAPK) and prevent it from activating any downstream transcription factors (Daynes and Jones, 2002). The PPARs have been shown to have mechanistic roles in both innate and adaptive immunity, leading to immune suppression. This is no surprise considering their expression in immune cells such as dendritic cells, T cells, B cells and macrophages as well as in epithelial cells (Straus and Glass, 2007). The *in vivo* role of PPARα activation in relation to infection is still unsure. Taken into account the described anti-inflammatory and immune repressing abilities, we first hypothesized that their up-regulated transcription was the main reason of the long-lived *Giardia* infections in cattle. This was further supported by the lack of immune response, inflammation or immune cell recruitment in the intestine of these animals. However, further work in mice infected with *G. muris* revealed that PPARα had no influence on infection duration since the administration of a PPARα agonist or the deletion of PPARα did not alter the speed at which the infection was cleared nor the ability of the animals to raise an IL-17A response. It does however appear that BALB/c animals show a stronger up-regulated transcription of *il17a* than seen in C57Bl/6 animals in combination with an absence of *ppara* up-regulation.

Intriguingly, recent studies have also shown a potential pro-inflammatory effect of PPARα ligands. This has been shown by the teams of Prof. K. De Bosscher and Prof. B. Staels (unpublished results) in a systemic TNF treatment-model where an unexpected pro-inflammatory effect of PPARα on liver cytokines was seen. Such pro-inflammatory role for PPARα has additionally been observed in a mouse model of endotoxemia in which dietary PPARα agonist treatment resulted in higher LPS-induced TNF plasma levels than in LPS-treated control-fed animals (Hill et al., 1999). A worsening of inflammation was also observed in a rat model of glomerulonephritis after treatment with a PPARγ agonist (Panzer et al., 2002). However, up until now, the effects of PPARs in relation to parasites have always appeared to be anti-inflammatory and immune suppressing (Chan et al., 2010). This is most likely so in the *Giardia* infected cattle, and considering the delay of IL-17A up-regulated expression until after the up-regulated expression of PPARα has disappeared, this also seems to be the case in mice.

The effect PPARα induction and activation might have on the host-parasite interaction stretches far beyond immune-modulation. The receptor plays an important role in the metabolic response to nutritional and environmental factors as is shown by its influence on the lipid metabolism (Varga et al., 2011). As a resident of the intestinal lumen, *Giardia* is subject to a variety of both host produced and dietary lipids. An important lipid for giardial growth which the parasite cannot produce *de novo* is cholesterol. The depletion of cholesterol, be it by epithelial absorption in the intestine or bacterial metabolisation, triggers the encystation of the trophozoites (Arguello-Garcia et al., 2009). Activation of PPARα reduces the absorption of luminal cholesterol by epithelial cells and induces higher excretion of the lipid via bile into the intestine (Valasek et al., 2007). One way PPARα executes its influence on cholesterol transport in the epithelium is by activating ATP-binding cassettes transporters. In chapter 2, the microarray analysis revealed an up-regulated transcription of ABCG8, a transporter that opposes the absorption of dietary sterols and promotes the excretion of cholesterol (Back et al., 2013). In addition, PPARα activation will decrease the amount of bile acids that are excreted in the intestine (Ito et al., 2013). These bile acids are important for the digestion of lipids in the intestinal lumen and would make cholesterol less available to the trophozoites (Arguello-Garcia et al., 2009). Theoretically, the higher abundance of lipids in the intestinal lumen would be beneficial for the parasite and could lower the need for encystation. This

could then explain the lower cyst excretion seen in infected C57Bl/6 mice compared to BALB/c animals (chapter 3). It this respect, it would be interesting to investigate the lipid content of faecal material of both infected and non-infected mice.

In production animals, changes in the metabolism of an animal could result in significant economical losses. Previous research on *G. duodenalis* infections in sheep has revealed that animals do suffer weight loss after infection (Olson et al., 1995), while experimental infection in goats described a decrease in appetite in infected animals (Koudela and Vitovec, 1998). Taking into account the modulatory role of PPARα on lipid and glucose metabolism, it is not unlikely that the nuclear receptor is related to the metabolic changes observed during *Giardia* infection. This has been shown already in rodents, where PPARα was related to weight loss, appetite suppression and satiety (Fu et al., 2003; Gutman et al., 2012; Perreault et al., 2010). In addition, diarrhea associated with a *Giardia* infection is often described as being mucoid or even fatty. This could again be explained by the increase of lipid content in the faeces after PPARα activation.

5.3. *Giardia* spp. and the peroxisome proliferator –activated receptor gamma

The question remains why in cattle it takes several months before IL-17A production can be seen after *G. duodenalis* infection, while in mice a protective response against *G. muris* is observed after 3 weeks with IL-17A up-regulation starting at 1 week *pi*. A possible cause of the different host responses to the different parasites could be the induction of PPARγ that was seen in cattle and not in mice. This subtype of the receptor has been directly linked to a suppression of IL-17 (Klotz et al., 2009; Park et al., 2009; Viladomiu et al., 2012). This regulatory function of PPARγ has been shown in both humans and mice end leads to a selective suppression of Th17 differentiation (but not the differentiation of T cells into Th1, Th2 or regulatory T cells) by controlling RORγt (Klotz et al., 2009).

Why activation of this receptor occurs in cattle and not in mice could be due to the difference in parasite species or the difference in host species. Interestingly, after being incubated with *G. duodenalis* for 18 hours, human intestinal Caco-2 cells showed an up-regulation of PPARγ, suggesting the same mechanism might also be active in humans infected with *G. duodenalis* (Roxstrom-Lindquist et al., 2005). To rule out the host species as a determining factor for the absence or presence of PPARγ

induction, it would be interesting to repeat the experiments using *G. duodenalis* in mice and again look at the transcriptional profile of the PPARs.

It should however be noted that *G. duodenalis* infections in mice often last only a short duration of time, with the elimination phase of an infection starting after around a month (Byrd et al., 1994). In this sense, it seems unlikely that PPARγ activation will be present in its role as advocator of an anti-inflammatory response and that a Th-17 response will be present. In addition, using this model raises the question if administration of *Giardia* in such high quantities and in a trophozoite form is a realistic representation of an infection in a natural host to *G. duodenalis*.

5.4. Conclusions and future prospects

In this thesis it became clear that calves infected with *G. duodenalis* do not mount any immune response to the parasite in the intestine during the first few weeks of infection but did show an activation of the PPARs. In *G. muris* infected C57Bl/6 mice, we could confirm an early up-regulation of PPARα but not PPARγ. No up-regulated transcription of any of these PPARs could be seen in BALB/c mice (although in the agonist trial, *ppara* up-regulation could be seen in the infected group at week 3 *pi*). After about one week of infection, a strong IL-17A response followed. This cytokine likely is crucial in the protection against the parasite, since IL-17RA KO animals were unable to clear infection in contrast to the infected WT mice.

Although *G. duodenalis* is not the most devastating pathogen in the intestine and often infections go by without overt inflammation of the intestinal tissue, it has been linked to production losses in ruminants. Clearing an animal of *Giardia* is not easy: treatment is difficult and does not prevent reinfection. Most animals will experience a new uptake of the parasite only shortly after treatment.

With this knowledge, the idea of a preventive measure such as a vaccine becomes more attractive. The purpose of a vaccine is to induce or stimulate a protective immunological memory reaction in the host to a foreign agent. The work presented in this thesis suggests that in the case of Giardia this is a Th17 like response.

It has been described before that certain vaccines require the induction of a memory Th17 response for the vaccine to be effective. Antigen specific Th17 inducing vaccination gave protection against *Bordetella pertussis* (Higgins et al.,

2006), *Mycobacterium tuberculosis* (Khader et al., 2007) and rotavirus (Smiley et al., 2007) in murine models. Most of their success laid in the use of the appropriate adjuvant. For IL-17 induction, adjuvantia such as dimethyl dioctadecylammonium bromide + monophosporyl lipid, fungal β-glycan end fungal hyphae have been described and tested successfully. Concerning the intestine specifically, systemic immunisation using lipopolysaccharide (LPS) as an adjuvant gave a potent intestinal Th17 priming, with high numbers of Th17 cells in the lamina propria of the murine intestine (McAleer et al., 2010). Although IL-17A production might be beneficial in the response to *Giardia,* it is still necessary to remain careful with the induction of an IL-17 response through vaccination. The role of IL-17A has been described as dual, on one hand IL-17A can be protective for the intestinal tissue, but on the other, uncontrolled production (as seen during autoimmune disease) can lead to tissue injury and damage. To fully understand how the production of IL-17A could give a safe protection against *Giardia*, we need more information on the onset and regulation of the IL-17A response.

Boosting the immune response through vaccination might also raise another issue. Consistent with the work presented in chapter 2, a recent development in the *Giardia* field are studies that report on an "immune-modulatory" effect of the parasite (Cotton et al., 2014), leading to immune suppression or a reduction/absence of inflammation in the intestine. In addition, work done in humans has revealed that the severity and incidence of diarrhea in children may be reduced by the presence of *Giardia* alone or as part of a co-infection with a diarrhea causing pathogen (Bilenko et al., 2004; Muhsen et al., 2013; Veenemans et al., 2011). However, other studies still link *Giardia* infection to clinical disease or do not see a beneficial effect on diarrhea of (co-)infection with *Giardia* (Bhavnani et al., 2012) . Here it could be questioned again if a difference in assemblage leads to a different response.

The microarray work done in the calves (chapter 2) revealed the PPARs as possible modulators of the absence of an immune response in the infected animals. The work done in *G. muris* mice questioned the role of PPARα in the regulation of the immune development. However, PPARγ is still an interesting candidate to solve the problem of immune suppression during a *Giardia* infection. As mentioned before, PPARγ has a direct and specific suppressing effect on IL-17A production. In this aspect, inhibition of PPARγ through the use of inhibitors or antagonist could enable the development of a protective Th17 response during infection. For now, PPARγ

antagonist have mostly been used in research concerning diabetes, obesity and cancer. The consequence of the use of these molecules on the entire body or in certain organs is not known. It should be noted that PPARγ, as is the case with PPARα, can be found in numerous types of cells and has a role in many different aspects of the metabolism. A specific example of the influence of PPARγ in the intestine is the maintenance of gut homeostasis. A study by Kelly et al. (2004) described that commensals used a PPARγ-dependent mechanism to dampen intestinal inflammation. With this knowledge in mind, additional research is needed on different aspects of PPARγ induction, regulation and functioning before it is feasible to temper with this receptor in the intestine.

5.5. References

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Summary

Summary

The protozoan parasite *Giardia duodenalis* is a highly present pathogen around the globe in a vast array of hosts. Once ingested, *Giardia* can induce intestinal symptoms (such as diarrhea, nausea and abdominal cramps) and could cause significant production losses in animals such as cattle. Although infections can regularly pass by unnoticed and rapidly, a number of patients experience giardiosis as a persistent and chronic disease. When looking at cattle, the chronicity is a given for any infection, with cyst excretions lasting well over 100 days. Although we have already some knowledge on the immune response in these animals and other natural hosts, the reason behind the observed chronicity is still unclear and *Giardia* induced host-pathogen interactions remain incompletely defined.

Therefore, the overall aim of this thesis was to unravel the intestinal host response in chronically infected cattle and to compare the results to what is happening in *G. muris* infected mice, in which the infections are short-lived. In this way, we attempted to identify the origin of the differences in infection duration.

The first step in the research presented in this thesis (chapter 2) was to investigate the intestinal response in calves following a *G. duodenalis* infection using a bovine high-density oligo microarray to analyze global gene expression in the small intestine. The resulting microarray data suggested a decrease in inflammation, immune response, and immune cell migration in infected animals. These findings were examined in more detail by histological analyses combined with quantitative real-time PCR on a panel of cytokines. The transcription levels of IL-6, IL-8, IL-13, IL-17, and IFN-γ showed a trend of being downregulated in the jejunum of infected animals compared to the negative controls. No immune cell recruitment could be seen after infection, and no intestinal pathologies, such as villus shortening or increased levels of apoptosis. Possible regulators of this intestinal response were the nuclear peroxisome proliferator-activated receptors alpha (PPARα), and gamma (PPARγ), all for which an upregulated expression was found in the microarray and qRT-PCR analyses.

In chapter 3, the objective was to gather further insights in the intestinal host response by using a murine giardiosis model where BALB/c and C57Bl/6 mice are infected with their natural parasite *Giardia muris*. Again, qRT-PCR was used to analyse

cytokine transcription patterns in intestinal tissue at different time-points during infection. In all mice, a strong up-regulation of *Il17a* could be observed starting around 1 week *post infection*. The significance of IL-17A in orchestrating a protective immune response was unequivocally demonstrated in an infection trial using IL-17 receptor A C57Bl/6 KO mice: whereas in wild type C57Bl/6 mice cyst excretion dropped significantly after 3 weeks of infection, the IL-17RA-KO mice were unable to clear the infection.

In chapter 4, the aim was to investigate the expression of the peroxisome proliferator-activated receptors in the intestine of *G. muris* infected mice, based on the work in chapter 2 where these receptors showed an up-regulated expression in *Giardia* infected calves. No up-regulation could be seen for PPARγ in both mice strains, and in addition, no up-regulated expression was seen for PPARα in BALB/c animals. Analysis of the intestinal response in C57Bl/6 mice however revealed a PPARα induction soon after the initial contact with the parasite, as characterized by the transcriptional up-regulation of *Ppara* itself and several downstream target genes such as *Pltp* and *Cpt1*. This induction of PPARα did not seem to have any influence on the immune response against *G. muris* since BALB/c mice administrated with a PPARα agonist and PPARα KO C7Bl/6 animals expressed *Il17a* and could clear the infection similar to WT controls.

Overall, we concluded that IL-17A is likely to be a crucial cytokine in the response to *Giardia* infection and the elimination of the parasite. In cattle it seemed that the PPARs were responsible for the lack of immune response because of their anti-inflammatory and immune suppressing characteristics, but this could not be clarified in *G. muris* infected mice when looking at PPARα. This nuclear receptor showed an up-regulation early on in infection but did not seem to influence infection duration. In future it will be interesting to look at the role of PPARγ in more detail as well as the ligands responsible for inducing the activation of the PPARs. In addition, future work can focus on the effector mechanisms induced by IL-17A that lead to the final elimination of the parasite from the host intestine.

Samenvatting

Samenvatting

De protozoaire parasiet *Giardia duodenalis* is een globaal sterk aanwezige parasiet met een grote waaier aan gastheren. Wanneer *Giardia* opgenomen wordt door een dergelijke gastheer, kan het gastro-intestinale symptomen veroorzaken (zoals diarree, misselijkheid en abdominale krampen) en de oorzaak zijn van significante productieverliezen in dieren zoals vee. Hoewel infecties regelmatig onopgemerkt en snel voorbijgaan zal in een aantal patiënten giardiosis een chronische en persistente ziekte zijn. In vee echter is chroniciteit zichtbaar bij elke infectie en kunnen cyst excreties nog waargenomen worden in de faeces na 100 dagen. Hoewel er reeds enige informatie beschikbaar is omtrent de immuun respons in deze dieren en andere natuurlijke gastheren, is de reden achter de waargenomen chroniciteit nog onbekend en zijn de *Giardia* geïnduceerde gastheer-parasiet interacties nog onvoldoende gedefinieerd.

Het doel van deze thesis was dan ook het verder ontrafelen van de intestinale immune response in vee en om de bekomen resultaten te vergelijken met *G. muris* geïnfecteerde muizen in dewelke infecties van korte duur zijn. Op deze manier hebben wij getracht om de oorsprong van de verschillen in infectieduur te identificeren.

De eerste stap in het onderzoek dat voorgesteld wordt in deze thesis (hoofdstuk 2) was het bestuderen van de intestinale respons in *G. duodenalis* geïnfecteerde kalveren met behulp van een boviene hoge densiteit oligo microarray waardoor de globale genexpressie in de dunne darm kon worden nagegaan. De bekomen microarray date suggereerde het ontbreken van inflammatie, immune response en immuun cel migratie in de geïnfecteerde dieren. Deze bevindingen werden in meer detail bestudeerd met behulp van histologische analyses gecombineerd met een kwantitatieve real-time PCR op een panel van cytokines. De transcriptie niveaus van IL-6, IL-8, IL-13, IL-17 en IFN-γ vertoonde een trend van neerregulatie in het jejunum van geïnfecteerde dieren in vergelijking met negatieve controles. Er werd geen rekrutering van immuun cellen gezien terwijl ook intestinale pathologische letsels, zoals het verkorten van villi of het verhogen van het aantal apoptotische cellen, afwezig bleven. Mogelijke regulatoren van deze intestinale response zijn de

peroxisoom proliferator-geactiveerde receptoren alfa (PPARα) en gamma (PPARγ), voor dewelke een op-gereguleerde expressie kon gezien worden op zowel de microarray data als de qRT-PCR analyses.

In hoofdstuk 3 werd een muis model gebruikt waarbij BALB/c en C57Bl/6 muizen geïnfecteerd werden met *G. muris* teneinde een beter inzicht te krijgen in de intestinale gastheer respons. Hierbij werd opnieuw qRT-PCR gebruikt om cytokine transcriptiepatronen te analyseren in het darmweefsel op verschillende tijdstippen tijdens de infectie. In alle muizen kon een sterke op-regulatie van *il17a* waargenomen worden vanaf ongeveer 1 week na infectie. Het belang van IL-17A in de bescherming tegen *G. muris* werd ontegensprekelijk aangetoond in een infectieproef met IL-17 receptor A C57Bl/6 KO muizen: terwijl in wildtype C57Bl/6 muizen cyst excretie sterk daalde na 3 weken infectie, waren de KO muizen niet in staat om zich te ontdoen van parasiet.

Hoofdstuk 4 had als doelstelling het onderzoeken van de expressie van de peroxisome proliferator-geactiveerde receptoren in *G. muris* geïnfecteerde muizen aangezien in hoofdstuk 2 de op-regulatie van de PPARs in *Giardia* geïnfecteerde kalveren werd waargenomen. Er kon geen op-regulatie waargenomen worden van PPARγ, noch in BALB/c noch in C57Bl/ 6 muizen. In de C57BL/6 muizen werd een PPARα inductie aangetoond kort na het eerste contact met de parasiet, zowel door de transcriptionele op-regulatie van PPARα zelf als van verscheidene downstream target genen zoals *Pltp* en *Cpt1*. In het algemeen lijkt PPARα geen invloed uit te oefenen op de immuunrespons tegen *G. muris* vermits in BALB/c muizen die een agonist toegediend krijgen en in PPARα KO dieren nog steeds een *Il17a* op-regulatie waargenomen kon worden en de dieren geen probleem bleken te hebben met het elimineren van de parasiet.

Uit deze thesis kan geconcludeerd worden dat IL-17A waarschijnlijk een belangrijke cytokine is in de respons tegen een *Giardia* infectie en de uiteindelijke eliminatie van de parasiet. Bij runderen leken de PPARs verantwoordelijk voor het ontbreken van de immuunrespons gezien hun anti-inflammatoire en immuun onderdrukkende eigenschappen, maar dit kon niet verder geruggesteund worden in *G. muris* geïnfecteerde muizen. Hoewel PPARα transcriptie op-gereguleerd na contact met de

parasiet, leek dit niet de duur van een infectie te beïnvloeden. Een interessante invalshoek voor verder toekomstig *Giardia* gericht onderzoek is het in meer detail bestuderen van PPARγ alsook van de verantwoordelijke liganden ter inductie en activering van de PPAR's. Bovendien kan verder onderzoek ook richten tot het ontrafelen van de door IL-17A geïnduceerde effectormechanismen die leiden tot de uiteindelijke verwijdering van de parasiet uit de dunne darm van de gastheer.

Appendix

Appendix

Primer sequences: cattle

Gene symbol	Accesion number	Primer sequence
Housekeeping genes		
ACTB	NM_173979.3	F: GACATCCGCAAGGACCTCTA R: ACATCTGCTGGAAGGTGGAC
GAPDH	NM_001034034.1	F: ACCCAGAAGACTGTGGATGG R: CAACAGACACGTTGGGAGTG
HPRT1	NM_001034035.1	F: CACTGGGAAGACAATGCAGA R: ACACTTCGAGGGGTCCTTTT
RPLP0	NM_001012682.1	F: CTTCATTGTGGGAGCAGACA R: GGCAACAGTTTCTCCAGAGC
SDHA	NM_174178.2	F: ACATGCAGAAGTCGATGCAG R: GGTCTCCACCAGGTCAGTGT
UBE2D2	NM_001046496.1	F: AGCCAGTTCTCCAGGCATAA R: TCTTCCCCATCCAAGAACAC
Validation microarray		
ABCG8	NM_001024663	F: GACCAGCATTGACAGACGAA R: CACTCTGCTCCCTTGACTCC
ADA	NM_173887.2	F: GACCTGGCTGGAGATGAGAC R: CTCGGTCTTGAGTGTGTCCA
FASN	NM_001012669.1	F:CTGAGTCGGAGAACCTGGAG R: CATATTGTGTGCCTGCTTGG
PPARA	NM_001034036.1	F:TCCCTCTTTGTGGCTGCTAT R: TCGTCAGGATGGTTGTTCTG
PPARG	NM_181024.2	F: GATCTTGACGGGAAAGACGA R: ACTGACACCCCTGGAAGATG
RASGRP2	NM_001099946.1	F: AGCCTGCTGATGGTGTCTTT R: GTTGGCGAGGACTTAGAACG
RHOD	NM_001192338.2	F: ATGTCACCAGTCCACACAGC R: TCACCAGCATCTTGTCCTTG
Cytokines		
IFN-γ	NM_174086.1	F: TTCTTGAATGGCAGCTCTGA R: TTCTCTTCGGCTTTCTGAGG
IL-1β	NM_174093.1	F: AAGGCTCTCCACCTCCTCTC R: TTTGGGGTCTACTTCCTCCA
IL4	NM_173921.2	F: GCGGACTTGACAGGAATCTC R: TCAGCGTACTTGTGCTCGTC
IL-6	NM_173923.2	F: TCCTTGCTGCTTTCACACTC R: CACCCCAGGCAGACTACTTC
IL-8	NM_173925.2	F: GTTGCTCTCTTGGCAGCTTT R: GGTGGAAAGGTGTGGAATGT
IL-10	NM_174088.1	F: TGTTGACCCAGTCTCTGCTG R: GGCATCACCTCTTCCAGGTA

Gene symbol	Accesion number	Primer sequence
IL-13	NM_174090.1	F: GGTGGCCTCACCTCCCCAAG R: ATGACACTGCAGTTGGAGATGCTG
IL-17	NM_001008412.1	F: GGACTCTCCACCGCAATGAG R: TGGCCTCCCAGATCACAGA
TGF-β1	NM_001166068.1	F: CTGCTGTGTTCGTCAGCTCT R: TCCAGGCTCCAGATGTAAGG
TNF-α	NM_173966.2	F: GCCCTCTGGTTCAGACACTC R: AGATGAGGTAAAGCCCGTCA

Primer sequences: mice

Gene symbol	Accession number	Primer sequence
Housekeeping genes (1)		
Actb	NM_007393.3	F: CTTCTTTGCAGCTCCTTCGTT R: TTCTGACCCATTCCCACCA
Gadph	NM_008084.2	F: CCTCGTCCCGTAGACAAAATG R: TGAAGGGGTCGTTGATGGC
Gusb	NM_010368.1	F: CCGATTATCCAGAGCGAGTATG R: CTCAGCGGTGACTGGTTCG
Hprt1	NM_013556.2	F: TGGATACAGGCCAGACTTTGTT RCAGATTCAACTTGCGCTCATC
Rplp0	NM_007475.5	F: ACTGAGATTCGGGATATGCTGT RTGCCTCTGGAGATTTTCGTG
Tbp	NM_013684.3	F: CAAACCCAGAATTGTTCTCCTT R:ATGTGGTCTTCCTGAATCCCT
qRT-PCR		
Camp	NM_009921.2	F: TCCCAAGTCTGTGAGGTTCC R: AGGCACATTGCTCAGGTAGC
Cpt1c	NM_153679.2	F: TCTTCACTGAGTTCCGATGGG R: ACGCCAGAGATGCCTTTTCC
Defb2	NM_010030.1	F: CCACACCAATGGAGGGTACT R: GGGGTTCTTCTCTGGGAAA
Defb3	NM_013756.2	F:ATTTCTCCTGGTGCTGCTGT R: TCCACAACTGCCAATCTGAC
Ifng	NM_008337.3	F: AGCTGCCATCGGCTGACCTA R: CCGCAGGAGGAGAAGCCCAGA
Il1b	NM_008361.3	F: GAAGAGCCCATCCTCTGTGA R: TCCATTGAGGTGGAGAGCTT
Il4	NM_021283.2	F: TGTACCAGGAGCCATATCCAC R: CACCTTGGAAGCCCTACAGA

Gene symbol	Accession number	Primer sequence
Il13	NM_008355.3	F: CTCAGCCTGCACTGCCTGCC R: GCTCAAGCTGCTGCCTGCCT
Il17a	NM_010552.3	F: CTACCTCAACCGTTCCACGT R: AGCTCTCAGGCTCCCTCTTC
Il17e	NM_080729.3	F: CAGTCCCAGTGACGCTAGAC R: ACCCACTAGAGCGGTGAGAG
Il17f	NM_145856.2	F: TGCTACTGTTGATGTTGGGAC R: AATGCCCTGGTTTTGGTTGAA
Pdk4	NM_013743.2	F: AGGGAGGTCGAGCTGTTCTC R: GGAGTGTTCACTAAGCGGTCA
Pltp	NM_011125.2	F:TGCTGAACATCTCCAACGCAT R: CACTTTAATCCGACCACTGGAAT
Ppara	NM_001113418.1	F:GCTGAAGCTGGTGTACGACA R:TGCCCAGAGATTTGAGGTCT
Pparg	NM_001127330.1	F:CACCAACTTCGGAATCAGCT R:AACCATTGGGTCAGCTCTTG
Reg3g	NM_011260.1	F:GCCCTCAGGACATCTTGTGT R: ACTCCCATCCACCTCTGTTG
Rorc	NM_011281.2	F:GAACCAGAACAGGGTCCAGA R:CGTAGAAGGTCCTCCAGTCG
S100a8	NM_013650.2	F:TCACCATGCCCTCTACAAGA R:GAGATGCCACACCCACTTTT
S100a9	NM_001281852.1	F:ACACCCTGAGCAAGAAGGAA R:GTCCAGGTCCTCCATGATGT
Tgfb1	NM_011577.1	F:TTGCTTCAGCTCCACAGAGA R:TGGTTGTAGAGGGCAAGGAC
Tnf	NM_001278601.1	F:ACGGCATGGATCTCAAAGAC R:GTGGGTGAGGAGCACGTAGT

1. **Wang, F., J. Wang, D. Liu, and Y. Su.** 2010. Normalizing genes for real-time polymerase chain reaction in epithelial and nonepithelial cells of mouse small intestine. Anal Biochem **399:**211-217.

Dankwoord
(Acknowledgements)

Er zijn heel wat mensen die ik graag wil bedanken voor de steun en de hulp tijdens de afgelopen jaren.

Allereerst bedankt aan Prof. Jozef Vercruysse voor de mogelijkheid om in het laboratorium voor parasitologie te doctoreren. Het is onvoorstelbaar hoe vaak we op uitstap zijn mogen gaan met het nodige lekker eten en drinken. Ook hiervoor merci!

Peter, bedankt voor de afgelopen 5 jaar, echt. Ik denk dat jij van dichtbij hebt kunnen meemaken dat mijn doctoraat mij niet alleen als wetenschapper maar ook als mens heeft doen groeien. Natuurlijk een dikke merci voor al de goede raad, de ideeën en het vertrouwen. Maar ook: voor al de keren dat er gewoon eens goed gelachen kan worden. Enneuh, hopelijk heb je geen al te groot trauma overgehouden aan het colaflesje of -blikje (=echt wel beter) verhaal.

Edwin, ik apprecieer het enorm dat je altijd de tijd nam om alles na te lezen en bedankt voor de interesse in mijn werk. Sorry dat ik nooit heb getrakteerd op echte Limburgse boekweitkoek.

Bedankt aan de overige leden van mijn examencommissie, Prof. Dr. Stefan Magez, Dr. Claude Libert, Prof. Dr. Karolien De Bosscher en Prof. Dr. Louis Maes voor het nalezen en verbeteren van mijn doctoraat en aan de voorzitter, Prof. Dr. Hans Nauwynck, om alles in goede banen te leiden. Karolien, bedankt voor de vele tripjes naar de faculteit diergeneeskunde om ons goede raad te geven.

Manuela, you were there at the very beginning of my PhD. Don't underestimate how much you gave me a push in the right direction.

Aan al mijn geweldige (ex)collega's: jullie zijn met teveel om op te noemen, maar ik ben echt iedereen dankbaar voor het leuke gezelschap en de hulp. Magda, bedankt om mij te helpen met al de muizen. Dirk, Mieke en Isabel, merci voor de hulp met allerlei praktische en administratieve zaken.

Stijn, jij moet een speciale vermelding krijgen. Jij bent één van de leukste mensen die ik ken en een enorm behulpzame collega.

Dries, Frederik and Tao: thanks for the fun moments, in Belgium and China.

Iris, merci dat ik zoveel met je heb kunnen babbelen over al dan niet werk gerelateerde zaken. Marijke, ben blij dat ik je mijn bureaugenote mag noemen. Sien (creatief genie) en Fiona: jullie zijn allebei toffe collega's waarmee ik graag aan de keukentafel rondhang. Ana, I've enjoyed that I could have the type of conversations with you after work that would make other people blush ;). Marvel has a movie plan that will ensure releases for several years to come…I'm sure many of these we will

watch together! Elise, mijn sushi-sister, altijd paraat “when shit hits the fan”. Kan ik je verleiden om mee te gaan naar hierboven vermelde films als ik wasabi-popcorn meeneem? Oonagh, ik heb er het volste vertrouwen in dat jij nog mooi onderzoek gaat doen op *Giardia*. Bruno en Johannes, jullie kunnen nog wel jullie mannetje staan tegenover dat vrouwelijk geweld, niet?

Nathalie en Charlotte, ik beschouw jullie al lang niet meer als mijn collega’s. Op de eerste plaats en meest van al zijn jullie mijn vriendinnen. We hebben zoveel leuke avonden doorgebracht, laten we die traditie gewoon voortzetten, ok? ;)

Rika, Veronique, Dominique en Melanie, de meisjes van onderzoek, wanneer plannen we ons volgende etentje? Bedankt ook aan mijn “oudste” vriendinnen, de meisjes uit Limburg, voor de vele verjaardagsfeestjes. En Elke en Kathleen, merci om helemaal tot hier af te komen!

Mijn broer Johnny, merci om er bij te zijn! You’re the best, brotherman! Niet stoppen met je zus te voorzien van leuke muziek! She needs it!

Bedankt ook aan mijn grootouders, Jean en Bertha, die regelmatig vanuit het verre Limburg naar Gent kwamen, en daarbij vaak in een treinpanne of file beland zijn. Bomma, bedankt voor de soepjes, de keutelkeskoek en het zo vaak “onder handen nemen” van mijn vuil appartement (ook al had ik het stiekem-naar mijn normen toch-al “mooi” gepoetst). Bompa, merci om altijd je best te doen om te luisteren naar mijn te snel geratel of toch minstens te doen alsof.

Herb, je weet wat je voor mij betekent he.

Mijn allerliefste ouders, papa en mama. Hoe kan ik hier nu voldoende beschrijven hoe dankbaar ik jullie ben? Hoe ik echt wel “the world’s greatest parents” heb? Altijd mijn grootste fans, hoewel jullie vaak vanop de eerste rij meegemaakt hebben hoe ik soms een beetje schrik kon hebben van het leven en onzeker was over mijn capaciteiten. Maar dit lieten jullie nooit toe en jullie gingen er tegenin met aanmoedigingen en een blind vertrouwen in mijn kunnen. En ik heb geen schrik meer. Waarom zou ik ook? I can be fearless want ik heb jullie. Gelukkige dertigste huwelijksverjaardag!

Leentje